Identification of new genes involved in hereditary steroid-resistant nephrotic syndrome using next generation sequencing and in vivo functional characterization in drosophila melanogaster

Sara De Almeida Gonçalves

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Doctoral Thesis of

PARIS DESCARTES University

Doctoral School of Bio Sorbonne Paris Cité

Sara DE ALMEIDA GONÇALVES

A dissertation submitted in accordance with the requirements for the award of the degree of PhD in Science

Identification of new genes involved in hereditary steroid-resistant nephrotic syndrome using next generation sequencing and *in vivo* functional characterization in *Drosophila melanogaster*

Defense date - 28th September, 2017

Jury panel

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To my father

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Abstract

Abstract

Nephrotic syndrome (NS) is a kidney disease characterized by disruption of the glomerular filtration barrier and the massive loss of proteins into the urine. Although in the majority of cases treatment with steroids leads to remission of the disease, in 15-20% of cases the disease is not responsive to this therapy and is classified as steroid-resistant nephrotic syndrome (SRNS). SRNS is a clinical condition with high morbidity leading to progressive renal failure as well as multiple metabolic and cardiovascular complications. Extensive research over the last 20 years has identified more than 40 SRNS causing genes that are crucial for function of the podocyte, a highly specialized kidney epithelial cell. However, the mutated gene is still unknown in about half of the familial cases. We have used exome sequencing to identify new genes mutated in SRNS. In order to prove the pathogenicity of the identified mutations we used the *Drosophila* model, assessing defects of fly viability and the structure and function of nephrocytes, podocyte like-cells. My thesis work consists of two projects.

Firstly, we identified biallelic mutations in a new candidate gene, *SGPL1*, encoding the sphingosine 1-phosphate lyase, in individuals presenting SRNS with facultative adrenal insufficiency, ichthyosis, neurological defects and immunodeficiency. SGPL1 is the main catabolic enzyme of sphingolipids, irreversibly degrading sphingosine 1-phosphate into phosphoethanolamine and hexadecenal. In flies, these mutations were shown to decrease viability, induce nephrocyte defects and lead to the accumulation of sphingoid bases due to the loss of SGPL1 catabolic activity. Together, these results indicate that the identified *SGPL1* mutations are pathogenic and cause a new syndromic form of SRNS.

Moreover, in a second project, we defined the contribution of homozygous mutations found in two different genes, *ADD3* and *KAT2B*, to a complex phenotype found in affected individuals from one consanguineous family. These individuals presented with neurological defects, cataracts, mild skeletal defects, cardiomyopathy and SRNS. *ADD3* encodes adducinγ, an F-actin capping protein that also links the actin cytoskeleton to the spectrin based membrane skeleton, while *KAT2B* encodes the lysine acetyltransferase 2B, mainly known for acetylation of histones and modulation of transcriptional programs. We found additional nonrelated patients carrying only biallelic *ADD3* mutations that presented a partially overlapping syndrome but with no cardiac or renal manifestations. In the *Drosophila* model we found that both *ADD3* and *KAT2B* mutations impaired fly viability and that the *ADD3* mutation also impaired fly motor function. However, only the *KAT2B* mutation induced functional defects in *Drosophila* heart and nephrocytes. Altogether, these results suggest that *ADD3* mutations are responsible for a neurological phenotype with facultative cataracts and skeletal defects while the *KAT2B* mutation induces heart and kidney defects.

These results highlight the *Drosophila* as a good *in vivo* model to test the pathogenicity of the mutations found in SRNS candidate genes.

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List of Abbreviations

List of Abbreviations

ABC	ATP-binding cassette
APOL1	Apolipoprotein L1
ARHGAP24	Rho-GTPase-activating protein 24
ARHGDIA	Rho GDP-dissociation inhibitor 1
ASMase	Acid sphingomyelinase
ATG9A	Autophagy related protein 9A
BDSC	Bloomington Drosophila Stock Center
bHLH	Basic Helix Loop Helix
C1q	Complement C1q
C3	Complement C3
CD2AP	CD2 associated protein
Cerases	Ceramidases
CerS	Ceramide synthases
CERT	Ceramide transfer protein
CHD2	chromodomain helicase DNA binding protein 2
ChIP	Chromatin Immunoprecipitation
CNS	Central nervous system
CoRL	Cells of renin lineage
CRB2	Crumbs homolog 2
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAD	Auto-regulatory domain
DAG	Diacylglycerol
DID	Diaphanous inhibitory domain
DMS	Diffuse mesangial sclerosis
DNMT	DNA methyltransferase
Dpp	Decapentaplegic
DZNep	S-adenosylhomocysteine hydrolase inhibitor 3-deazaneplanocin A
EM	Electron microscopy
EMS	ethylmethanesulfonate
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
EZH2	Enzyme enhancer of zeste homolog 2
FGF	Fibroblast growth factor
FIB-SEM	Focused ion beam-scanning electron microscopy
FSGS	Focal and segmental glomerulosclerosis
GAP	GTP activating factors
Gb3	Globotriaosylceramide
GBM	Glomerular basement membrane
GDIs	Guanine dissociation inhibitors
GEFs	Guanine nucleotide exchange factors
GFB	Glomerular filtration barrier
GFP	Green fluorescent protein
GLA	Alpha-galactosidase A
Grb2	Growth factor receptor-bound protein 2

GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEK	Human embryonic kidney
IgM	Imunoglobulin M
INF2	Inverted formin 2
IQGAP	Iq motif containing GTPase-activating protein
JNK	c-Jun N-terminal kinases
JUNK	Jun-N-terminal protein kinase
KANK2	KN motif and ankyrin repeat domain-containing protein 2
KAT	Lysine acetyl-transferase
KAT2A	Lysine acetyl transferase 2A
KAT2B	Lysine acetyl transferase 2B
KD	Knock down
KDAC	Lysine deacetylase
Kirre	Kin-of-irre
KO	Knock out
LOF	Loss of function
LPS	Lipopolysaccharide
MAGI2	Membrane-associated guanylate kinase 2
MCD	Minimal change disease
mDia	Mammalian diaphanous related formin
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MEFs	Mouse embryonic fibroblasts
mTOR	Mammalian target of rapamycin
MYO1E	Myosin 1E
NGS	Next generation sequencing
NS	Nephrotic syndrome
Ntrk3	Neurotrophic tyrosine kinase receptor, type 3
N-WASP	Neural Wiskott-Aldrich-syndrome
PAK	p21 activated kinase
PAN	Puromycin aminonucleoside nephrosis
PCAF	P300/CBP associated factor
PDGF	Platelet derived growth factor
PE	Phosphoethanolamine
PECs	Parietal epithelial cells
PHB	Prohibitin
PI3K	Phosphoinositide 3-kinase
PP2A	Protein phosphatase 2A
PTM	Post translational modifications
PTPRO	Protein tyrosine phosphatase, receptor type O
RFP	Red fluorescent protein
RNAi	RNA interference
RPA	Replication protein A
S1P	Sphingosine 1 phosphate
S1PR	S1P receptors
SGPL1	sphingosine 1 phosphate lyase 1
SIOD	Schimke Immuno-Osseous Dysplasia

SMARCAL1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, subfamily A-Like 1
SMases	Sphingomyelinases
SMPDL-3b	Sphingomyelinase-like phosphodiesterase 3b
Sns	Sticks-and-stones
Sparc	Secreted protein acidic and rich in cysteine
SPHK	Sphingosine kinases
SPNS2	Spinster 2
SRNS	Steroid-resistant nephrotic syndrome
SSNS	Steroid-sensitive nephrotic syndrome
SUPAR	Soluble urokinase plasminogen activator receptor
TEM	Transmission electron microscopy
TRPC6	Transient receptor potential channel, subfamily C, member 6
UAS	Upstream activating sequence
VDRC	Vienna Drosophila Resource Center
VEGF	Vascular endothelium growth factor
WAVE	WASP-family verprolin-homologous protein
WES	Whole exome sequencing
Wg	Wingless
WGS	Whole genome sequencing
WT1	Wilms tumour 1
ZO-1	Zonula occludens-1

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Foreword

Foreword

In the last twenty years, genetic studies on familial forms of steroid-resistant nephrotic syndrome (SRNS) have allowed the identification of mutations in more than 40 genes that are crucial for the function of the podocyte, a highly specialized epithelial cell that lies in the glomerular filtration barrier (GFB). However, in more than half of the familial cases of SRNS, the underlying molecular defect is still unknown. The era of next-generation sequencing (NGS) brought new opportunities for the cases where a molecular diagnosis had not been established, however it also came with new challenges, in particular to address the functional impact of multiple rare and private variants. In this thesis we have used the *Drosophila* model to answer this need. Its short life cycle and the availability of advanced genetic tools make it an attractive model for studying the pathogenicity of mutations found in new candidate genes. Moreover, flies have nephrocyte cells which are homologous to podocytes, allowing us to specifically address the impact of the mutations found in SRNS patients.

In the introductory chapter of this thesis I start by detailing the general physiology and structure of the GFB and particularly of the podocyte, and reviewing the concept of SRNS as well as the mechanisms known to be involved in the podocyte response to injury and progression of glomerular disease. I then briefly review the structure of the podocyte slit-diaphragm, a modified cell junction that allows maintenance of the permselectivity of the GFB, and the podocyte actin cytoskeleton. This is followed by the genetics of the podocytopathies with a focus on the syndromic forms of the SRNS. I then present the advances and pitfalls of NGS techniques and the main advantages of the *Drosophila* model, focusing on the nephrocytes and their similarities to podocytes. I finish the introduction by exposing two novel thematics related to the two SRNS candidate genes discovered during this thesis: the complex sphingolipid metabolism and functions, and the fine-tuning of the DNA genomic information conferred by the epigenetic machinery. In both cases I give a special emphasis to the relationship of these mechanisms to the podocyte.

The results and discussion chapter is divided into two main parts, each focusing on one of the two different projects of this thesis. For each project, results are presented in the form of a journal manuscript and a summary of the findings and discussion then follows. In the first project we have identified mutations in *SGPL1*, encoding sphingosine -1-phosphate lyase, as a cause of a new syndromic form of SRNS, where the main extra-renal features were adrenal insufficiency, ichthyosis, immunodeficiency and neurological defects. We defined the pathogenicity of the mutations and started to address the impact of SGPL1 loss of function on the metabolism of sphingolipids and on the function of the nephrocytes. In the discussion I mainly focus on the phenotypic heterogeneity of

the disease and the possible underlying pathogenic mechanisms in the affected organs. In the second project we have defined the pathogenicity of biallelic mutations found in *ADD3* and *KAT2B* in affected individuals from one consanguineous family presenting a complex syndromic form of nephrotic syndrome. I discuss the contribution of *ADD3* and *KAT2B* mutations to the patient phenotype, the potential pathogenic mechanisms in the podocyte, as well as the difficulties that we found during the project.

This thesis will finish with a brief conclusion that highlights the great advantages but also the pitfalls that can be encountered when using the *Drosophila* model in human genetics.

All the work in this thesis was done under the direction of Corinne Antignac, my thesis supervisor, and Matias Simons, who shared with us his expertise on the *Drosophila* model and with whom we closely collaborated.

Review of the Literature

The glomerulus and the glomerular filtration barrier

The main role of the kidney is the regulation of the hydric, acid-base and ion balance and excretion of toxic compounds produced by cell metabolism. To achieve these functions the kidney depends on small functional structures called nephrons, which number up to ~$1x10^6$ per human kidney. Each nephron is composed of a glomerulus (**Figure 1**), responsible for the plasma filtration, and tubules that further modify this primary filtrate through secretion and reabsorption of small molecules, electrolytes and water, to finally produce urine.

Each day the kidney filters approximately 180 L of plasma. Blood enters the glomerulus through a small pre-glomerular arteriole (afferent arteriole) that then branches into a series of capillaries that form the glomerular capillary tuft. These then join again in another arteriolar, post-glomerular, vessel (efferent arteriole). This specialized vascular bed allows the fine-tuning of hydrostatic pressure inside the capillaries, the main driving force for filtration. Blood is thus filtered across the glomerular filtration barrier (GFB) to produce the primary urine.

The GFB has three layers. The first is formed by the fenestrated endothelium of glomerular capillaries. The meshwork-like glomerular basement membrane (GBM) forms the second layer, while the final layer is formed by specialized epithelial cells, termed podocytes, which extend foot processes that interdigitate and organize into a network that covers the outer surface of the capillary loops (**Figure 1**).

The main function of the GFB is to maintain permselectivity. This means that in normal homeostatic conditions the GFB is able to freely allow the passage of water, ions, by-products of cellular metabolism and small proteins but not blood cells or high molecular weight proteins. All three layers participate in this function (Kalluri 2006). The fenestrated endothelial cells prevent the passage of the blood cellular elements and the negative charge of the endothelial glycocalyx impedes the filtration of negatively charged molecules. Moreover the GBM dense meshwork also constitutes an obstacle to the passage of high molecular weight proteins. The GBM is around 300 nm in thickness, and is mainly composed of laminins, collagen IV, agrin and nidogen (Suh and Miner 2013, Lennon, Randles et al. 2014). Podocytes make the final layer and play a key role in GFB permeability. Interestingly, it has been proposed that filtration itself generates a local electric field at the GFB that also prevents the plasma proteins from entering or crossing the glomerular filter, explaining why the GFB does not clog (Moeller and Tenten 2013).

Besides the endothelial cells and podocytes, mature glomeruli also contain mesangial cells which are embedded in the mesangial matrix and are classically known to contribute to the structural integrity

of the capillary loops and to participate on the regulation of glomerular hemodynamics. Moreover, lining the glomerular Bowman's capsule, we find the parietal epithelial cells for which new roles have been ascertained in recent decades (**Figure 1**).

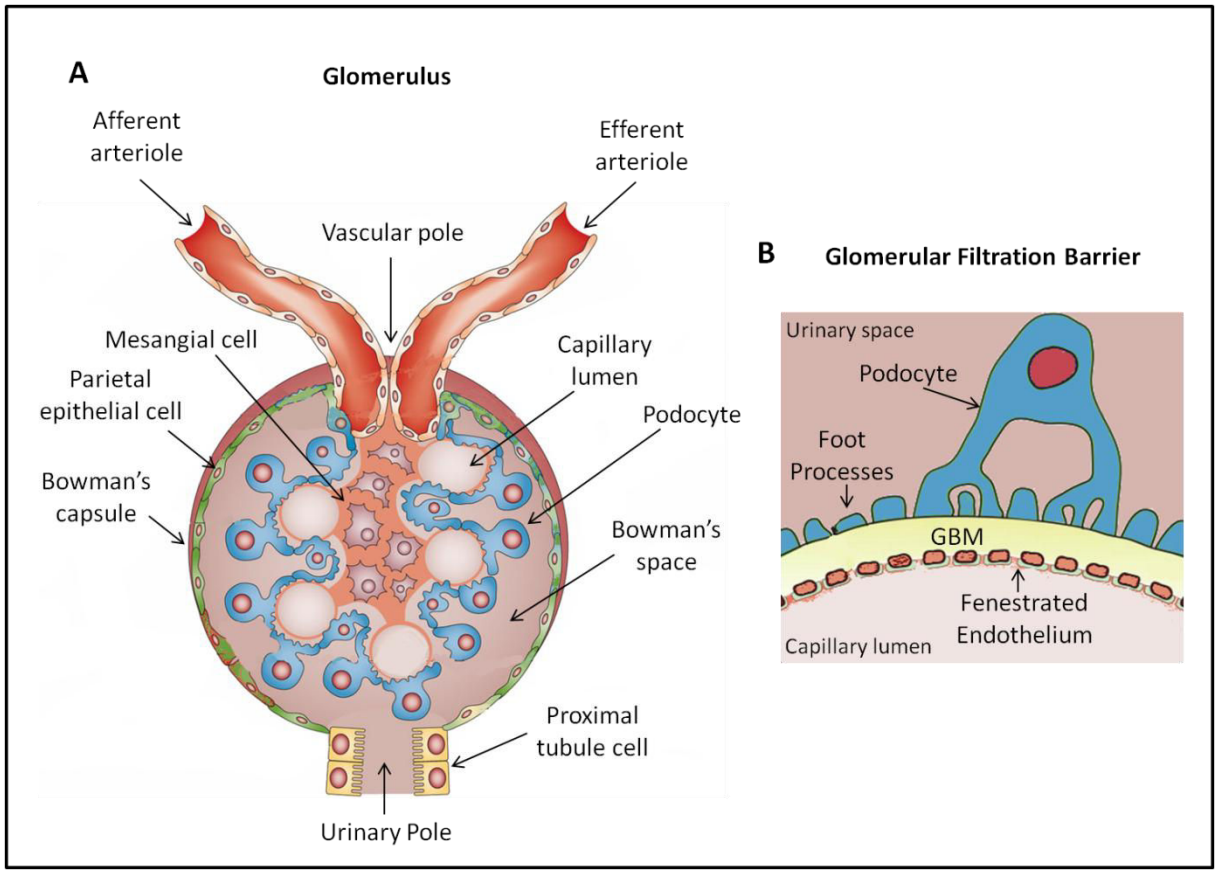

Figure 1. The glomerulus and the glomerular filtration barrier. (A) Structure of the glomerulus. The glomerulus contains a capillary tuft that receives its blood supply from the afferent arteriole. Podocytes line the outer aspect of the capillaries and their cell bodies float in the Bowman's space. Mesangial cells are embedded in the mesangial matrix and provide structural support to the capillary tuft. The Bowman capsule delimitates the glomerulus and is lined by parietal epithelial cells. **(B)** The glomerular filtration barrier. Plasma is filtrated through the glomerular filtration barrier that is composed of three layers: the fenestrated endothelial cells, the glomerular basement membrane (GBM) and the podocytes. Podocyte foot processes encircle the capillary loops. Adapted from Shankland, *et al*. 2014 and Patrakka *et al.* 2010.

The space between the glomerular tuft and the Bowman's capsule is named the urinary or Bowman's space, and corresponds to the compartment into which the plasma filtrate (also called primary urine) first passes. Low molecular weight proteins that pass the GFB are then reabsorbed in the first segment of the nephron tubule, the proximal tubule, by epithelial cells with dense microvilli at their apical surface.

The podocyte

Podocytes are highly specialized epithelial cells that sit in the GBM, wrapping around the capillary loops. Podocytes attach to the outer aspect of the GBM mainly by integrins (α3β1 integrin that binds to laminin and integrin-α1β1 and α2β2 that bind to type IV collagen) (Mathew, Chen et al. 2012) but also through α and β dystroglicans that link podocyte actin to GBM laminin, and syndecans (Perico, Conti et al. 2016).

Podocytes have a large cell body that floats in the urinary space and elongated cellular extensions called the podocyte processes (**Figure 2**). The first thick elongations are called the primary processes and their cytoskeleton is mainly composed of microtubules. These further divide into secondary processes, from which actin-rich foot processes project. Podocyte foot processes completely encircle the capillary loops and are linked to their neighbors by slit diaphragms, a modified adherens junctions (Tryggvason, Patrakka et al. 2006). The slit diaphragm is classically viewed as a zipper-like structure with pores that allow the passage of water and small solutes but limit the passage of large molecular weight proteins (Gagliardini, Conti et al. 2010). An anionic glycocalix composed of glycosaminoglycans and sialoproteins, including podocalyxin, covers the podocyte surface, is important for maintaining the structure of the foot processes (Kerjaschki, Sharkey et al. 1984, Pavenstadt 1998) and has recently been shown to also play a role in the size selectivity of the GFB (Lawrence, Altenburg et al. 2017).

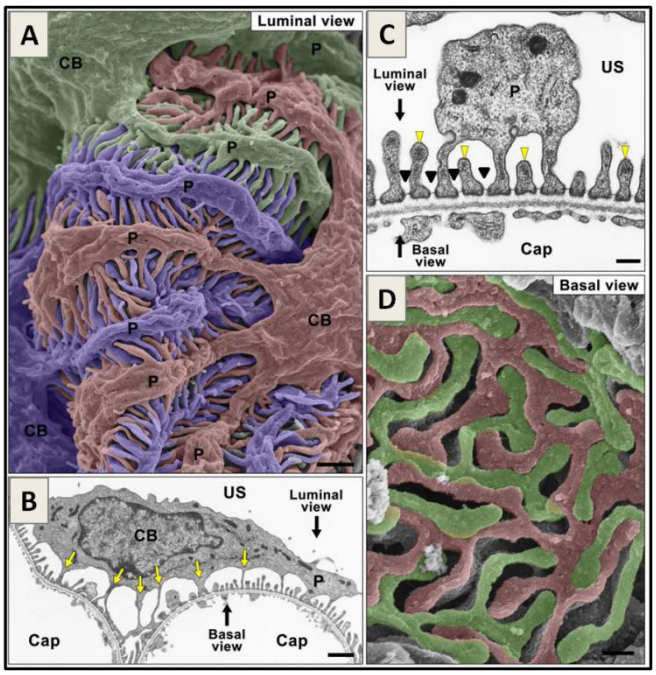

Figure 2. Ultrastructure of the podocyte. Podocytes are structurally divided in cell body (CB), primary and secondary processes (P) and foot processes. **(A)** Scanning electron microscopy images showing the CB, P and foot processes. Note that foot processes interdigitate with neighboring foot processes from another cell (individually colored in purple, green and red). **(B and C)** Transmission electron microscopy images **(B)** foot processes encircle the capillary loops (Cap). Arrows point to foot processes directly protruding from the podocyte CB. **(C)** Note the presence of a thin grey line between neighboring foot processes, corresponding to the slit diaphragm (black arrow heads), and the electron dense actin-bundle in the apical cytoplasm of foot processes (yellow arrow heads) **(D)** SEM images of the basal surface of podocytes. Bar scales, 1 µm in (A), (B); 200 nm in (C), (D). US, urinary space. Adapted from Ichimura *et al.* 2015.

Podocytes have to withstand the shear stress from the flow of filtrate and are continuously exposed to potential toxins from the plasma filtrate. This particular situation demands high plasticity and dynamics that depend on a finely regulated cytoskeleton. In fact podocytes express mechanosensors that can respond to physical stimuli like shear stress (Huber, Schermer et al. 2007). These cells have several other important functions, namely to synthesize many extracellular matrix components of the GBM, such as type IV collagen; to help to physically support the capillary loop; and to interact with other glomerular cells, namely endothelial cells and mesangial cells, in order to guarantee their normal function. A paradigmatic example of cell to cell interaction is the production by the podocytes of the soluble vascular endothelial growth factor (VEGF) that traverses the GBM counter-flow in order to act on VEGF receptors on the endothelial cell, *conditio sine qua non* the fenestrated endothelial cells swell and detach from the GBM as nicely shown by S. Qwaggin group, using a conditional KO of *Vegf* in podocytes (Eremina, Sood et al. 2003, Eremina, Jefferson et al. 2008). The same dependence on VEGF produced in podocytes has also been shown for mesangial cells (Eremina, Cui et al. 2006). Mesangial cells are also regulated by podocyte production of PDGF (van Roeyen, Eitner et al. 2011).

The nephrotic syndrome

Any disruption of the filtration barrier leads to loss of glomerular permselectivity and urinary protein loss. When the loss of proteins is massive, this leads to secondary alterations in plasma protein levels and their turnover, alterations in lipid metabolism, endocrine disturbances and hemostasis defects. Nephrotic syndrome (NS) is thus defined as the constellation of the following clinical findings: massive proteinuria (>3.5 g/day), hypoalbuminemia (<3.0 g/dL), its clinical symptom - edema, and hypercholesterolemia (Nishi, Ubara et al. 2016). NS is a condition which carries high morbidity due to cardiovascular complications, such as thrombosis, increased infection risk and malnutrition. Moreover, when NS persists and does not respond to therapy this leads to progressive scarring of the glomeruli, a process called glomerulosclerosis, and secondary fibrosis of the tubular-interstitial compartment. Glomerulosclerosis will ultimately lead to renal failure and end-stage renal disease (ESRD), and patients will need dialysis or kidney transplant to survive. Glomerular diseases leading to secondary glomerulosclerosis are a major health problem in the Occidental world and account for ≈85% of all ESRD (Tharaux and Huber 2012).

Extensive research over the past decades has highlighted the main role of the podocyte in NS. In fact, although Mendelian cases of NS are very rare (Boyer O 2016), genetic studies have made a major

contribution to this field with the identification of mutations in genes encoding proteins crucial for podocyte function. Moreover, the ultrastructural phenotype common to all NS forms is podocyte foot process effacement with slit diaphragm disruption (**Figure 3**).

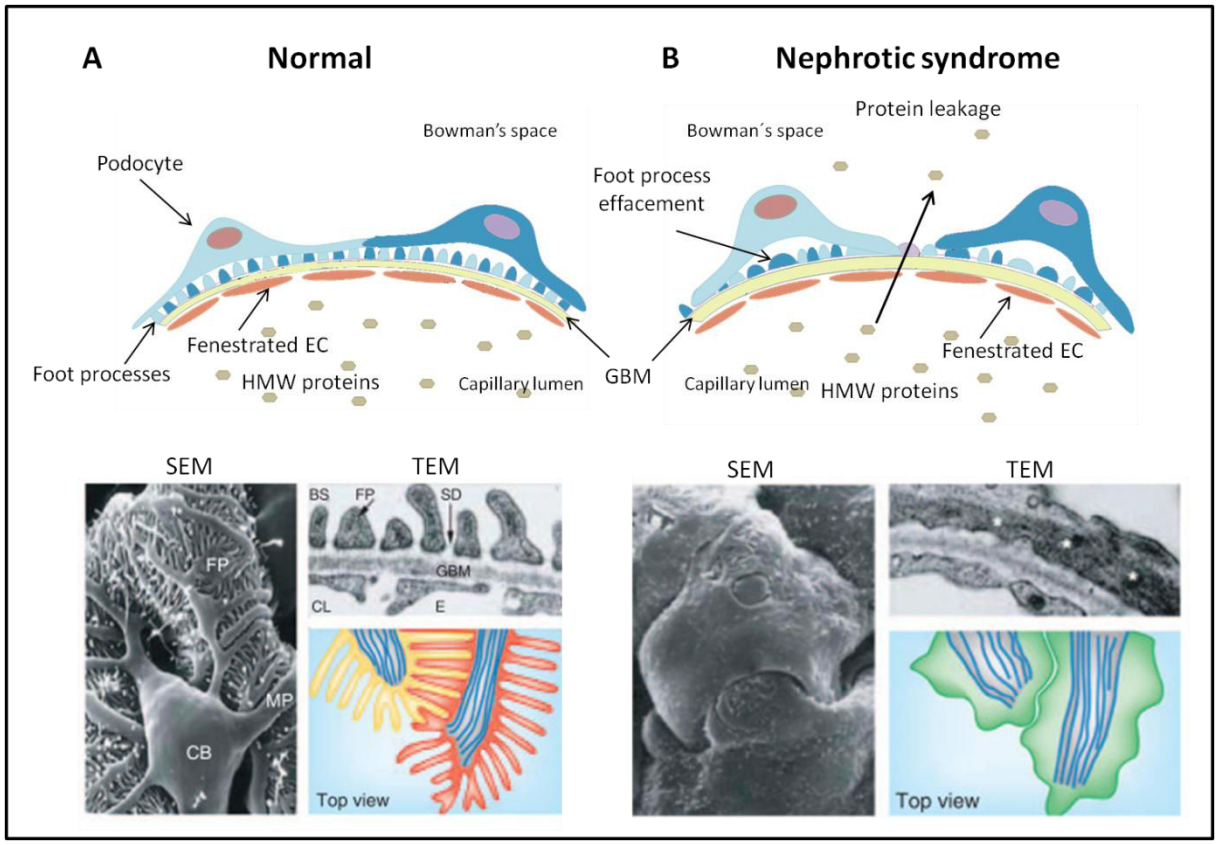

Figure 3. Podocyte structure in physiological conditions and upon injury. (A) Upper panel: In physiological conditions the GFB, composed of fenestrated endothelial cells (EC), GBM and podocytes, is able to prevent the spill of proteins into the urine. Lower panel: SEM and TEM images showing healthy podocytes and foot processes (FP), as well as the slit diaphragm (SD) and fenestrated endothelium (E) and a Schematic of normal podocyte foot processes **(B)** Upper panel: In nephrotic syndrome there is podocyte foot process effacement and protein leakage into the urine. Lower panel: SEM and TEM images showing podocyte foot process effacement and loss of SD. The corresponding schema shows a continuous sheet of cytoplasm instead of the well defined foot process. HMW, high molecular weight; BS, Bowman's space; CL, Capillary lumen. Adapted from Lin *et al.* 2016 and Mundel *et al.* 2010.

The process of foot process effacement depends on the rearrangement of the podocyte cytoskeleton in response to noxious stimuli and some authors consider that it may have developed as a mechanism to improve the attachment of these cells to the underlying GBM (Kriz, Shirato et al. 2013). If the noxious stimulus is transient, podocytes can recover their morphology and foot processes, as it occurs in self-remitting or steroid-sensitive NS. However, if the injury is persistent and/or severe enough, foot process effacement is followed by detachment of the podocyte foot processes from the GBM, the formation of pseudocysts and ultimately the detachment of these cells into the Bowman space and the nephron tubules, with resultantly high numbers of podocytes detectable in the urine (Kriz, Shirato et al. 2013). *In vivo* studies in the rat model have shown that

areas of the GFB with damaged podocytes seem to have high permeability to proteins (Peti-Peterdi and Sipos 2010). The ultrastructural changes have been clearly described by Kriz et al. (Kriz, Shirato et al. 2013) and are summarized in **Figure 4**.

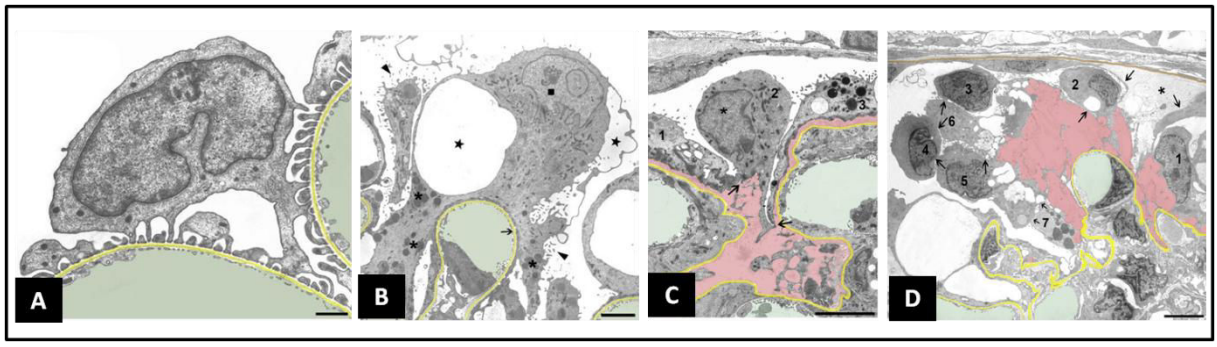

Figure 4. Ultrastructural changes accompanying podocyte injury and detachment. GBM is highlighted in yellow, capillary lumens in green and spaces between GBM and detaching podocytes in pink **(A)** Normal podocytes **(B)** Podocyte effacement in response to injury, formation of pseudocysts (stars) and microvillous transformation of the podocyte apical membrane (arrow head) **(C and D)** Podocyte detachment leaving areas of bare GBM. Bar scales, 1 µm in (A) 3 µm in (B and C); 5 µm in (D). Adapted from Kriz *et al.* 2013.

NS may be a primary or secondary glomerular disorder, in the latter case associated with metabolic disorders such as diabetes, infections (i.e. HIV, HBV, HCV), immune disorders (i.e. systemic lupus erythematosus) drugs (pamidronate, interferon), toxins or malignancy (Eddy and Symons 2003). Among primary disorders, idiopathic NS is characterized by the absence of inflammation and immune deposits in biopsy samples and is most commonly found in children but can also be found in adults. According to the age of apparition, NS can be further classified in congenital NS (<3 months); infant (3 months–12 months); early childhood (13 months–5 years); late childhood (6–12 years); adolescent (13–18 years); and adult (>18 years old) (Brown, Pollak et al. 2014).

Idiopathic NS has two main pathophysiological mechanisms: (1) a structural defect of the GFB, as it occurs in genetic diseases, and which will be the object of this thesis; (2) an immune defect believed to be a circulating permeability factor of lymphocyte origin that secondarily leads to podocyte damage (Antignac 2002) (**Figure 5**). In both cases the clinical presentation of the disease is similar but some differences allow their distinction: the presence of other affected organs, and the onset before one year of age, all suggest a genetic defect (Hinkes, Mucha et al. 2007), while response to immunosuppressive therapy and early relapse after kidney transplant suggest an immune defect (Ding, Koziell et al. 2014).

Whatever the underlying defect, idiopathic NS is always initially treated with a course of corticoids and, depending on the patients' response to this immunosuppressive therapy, it can be further classified into steroid-sensitive NS (SSNS) – if symptoms completely remit – or steroid-resistant NS (SRNS) – if symptoms persist. SSNS accounts for 80-90% of idiopathic NS and usually does not result

in renal failure. SRNS, which represents 10-20% of idiopathic NS, shows a worse prognosis progressing to ESRD if no response is obtained with other classes of immunosuppressors (Cattran and Rao 1998). Though SSNS and most SRNS cases are immunological in nature, a small percentage of SRNS cases have a genetic/structural cause (**Figure 5**) (Hinkes, Mucha et al. 2007, Ding, Koziell et al. 2014, Sadowski, Lovric et al. 2015, Boyer O 2016).

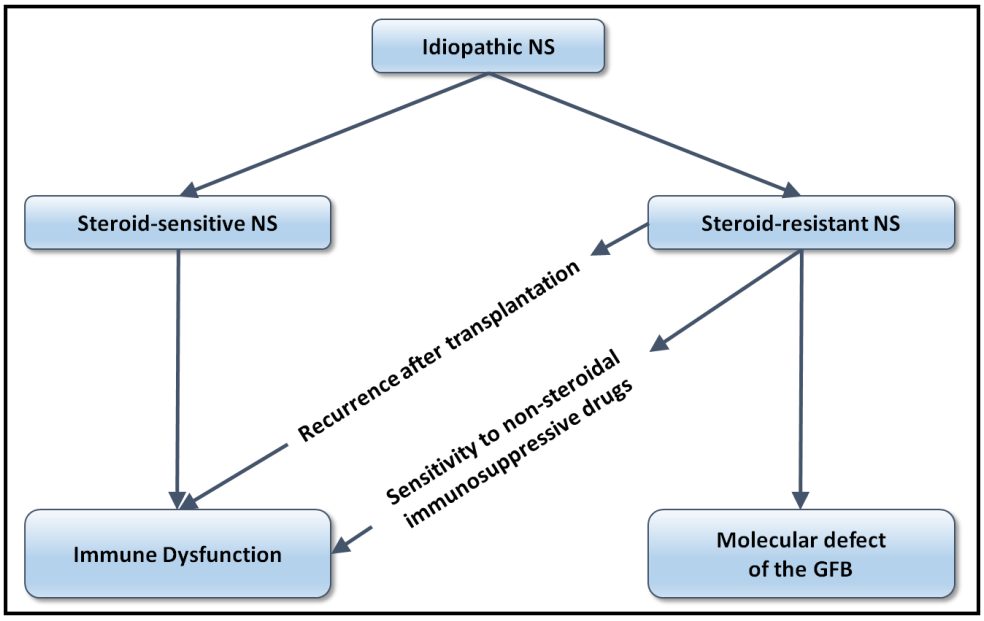

Figure 5. Mechanisms underlying idiopathic nephrotic syndrome. Immune dysfunction underlies steroid-sensitive forms and a subset of steroid-resistant forms, namely those that respond to treatment with other immunosuppressive drugs and those that recur after transplantation. The subset of SRNS patients that have an underlying molecular defect typically do not respond to any immunosuppressive drug and do not recur after transplantation. Adapted from Antignac *et al.* 2002.

Immune forms of nephrotic syndrome: the circulating permeability factor

Several lines of evidence suggest that an immune defect is the cause of most cases of SSNS and of SRNS relapse after transplantation, these include the response to immunosuppressive therapy, the NS relapse after infectious or allergic stimuli and the occurrence of NS with Hodgkin's disease, non-Hodgkin's lymphoma, or thymoma. Initial studies suggested that the immune defect was of T-lymphocyte origin (Shalhoub 1974). In fact, decreased T-regulatory cells and altered cytokine profiles have been identified in children with idiopathic NS (Araya, Diaz et al. 2009, Le Berre, Bruneau et al. 2009, Prasad, Jaiswal et al. 2015). The role of B-cells has also been suggested due to the efficacy of treatment with the monoclonal anti-CD20 antibody in affected individuals with steroid-dependent or frequent relapsing NS, however their exact role in the pathogenesis of the disease remains unknown (Iijima, Sako et al. 2014). Recently, hematopoietic stem/progenitor CD34-positive cells transferred from humans with idiopathic NS to immunocompromised mice have been shown to cause

proteinuria and podocyte effacement after three weeks (Sellier-Leclerc, Duval et al. 2007). However, the precise immune dysfunction mechanism remains unknown.

Several findings support the existence of a circulating permeability factor of immune origin, such as the proteinuria recurrence after kidney transplantation which occurs in about 30% of SRNS cases. This can occur within minutes to hours, and can be temporarily treated with plasmapheresis and immunoadsorption (Hoyer, Vernier et al. 1972). Studies in the rat model and case reports from humans have shown that when recurrence occurs following a kidney transplant and the organ is then grafted back to another recipient (or another rat strain), no proteinuria develops (Le Berre, Godfrin et al. 2002, Gallon, Leventhal et al. 2012). Moreover, kidneys from patients with SSNS that are transplanted into a patient with another type of kidney disease do not have recurrence of proteinuria (Gallon, Leventhal et al. 2012). In addition, serum from patients with NS is able to induce podocyte defects and proteinuria when injected into rats (Zimmerman 1984).

Several candidates have been proposed as circulating permeability factors. In cases of segmental glomerulosclerosis and chronic kidney disease, soluble urokinase plasminogen activator receptor (SUPAR) seemed to be the main candidate (Wei, El Hindi et al. 2011, Hahm, Wei et al. 2017). However, others have contested these findings and shown that high plasma SUPAR levels are mainly dependent on renal function (Meijers, Maas et al. 2014) and are a biomarker of cardiovascular disease (Lyngbaek, Marott et al. 2013, Meijers, Poesen et al. 2015), reflecting subclinical immunological activity and inflammation, but are not a mediator of a single disease entity (Meijers and Sprangers 2014).

Histologic variants of idiopathic nephrotic syndrome

In idiopathic NS three main types of non-specific histological lesions can be identified: minimal change disease (MCD), focal and segmental glomerulosclerosis (FSGS) and diffuse mesangial sclerosis (DMS). MCD and FSGS in particular have been proposed to correspond to two different morphological manifestations of the same underlying pathogenic process of podocyte injury (Maas, Deegens et al. 2016). These authors propose that there are several factors that will determine whether or not MCD will later evolve into FSGS lesions, including extent of injury, vulnerability of podocytes, other potential noxious factors and the response to therapy. In fact, clinical data suggest that patients with NS that are unresponsive to therapy or have a known molecular defect of genetic origin, can initially have findings of MCD by biopsy, and later develop FSGS (Tejani, Phadke et al. 1985). Moreover, the dose dependent development of FSGS lesions in the puromycin

aminonucleoside nephrosis (PAN) model and in other models of transient proteinuria, support the hypothesis that MCD and idiopathic FSGS are different manifestations of the same underlying injury and related to the severity of podocyte loss (Pippin, Brinkkoetter et al. 2009). This of course does not exclude the fact that several and not a single cause of podocyte injury exists.

Rodent models used for MCD and FSGS aim to induce podocyte injury and proteinuria. While in MCD the objective is to obtain a response that is transient and mild, FSGS lesion models are severe or prolonged enough to lead to the scarring process. Podocyte injury can be induced by antibodies directed against podocyte epitopes (endogenous or ectopically expressed) or by toxic agents. The last ones include the adriamycin and PAN models, protamine sulfate, and many others (Frenk, Antonowicz et al. 1955, Bertani, Poggi et al. 1982, Diamond and Karnovsky 1986, Leeuwis, Nguyen et al. 2010).

Minimal Change Disease

The pathological features include normal glomeruli on light microscopy and absence of immune deposits by immuno-fluorescence but the finding of widespread foot process effacement on electron microscopy. Clinically this lesion is associated to a higher rate of disease remission in response to corticotherapy and maintenance of kidney function (Mason 2010).

Focal and Segmental Glomerulosclerosis

FSGS is characterized by the sclerosis of a subset of the glomeruli (focal) and a portion of the glomerular tuft (segmental). Histologically a segmental obliteration of the glomerular capillaries by extracellular matrix can be observed (D'Agati 2008), as well as hyalinosis, corresponding to the accumulation of entrapped plasma proteins. Cellular adhesions, also called synechiae, can be formed between the Bowman capsule and the sclerosing segment. By electron microscopy, in addition to podocyte foot process effacement, regions of podocyte detachment can be observed. Immuno-fluorescence can show IgM and C3 deposits, corresponding to plasma proteins entrapped in areas of hyalinosis. With progression of the disease a more global and widespread glomerulosclerosis can be observed (D'Agati, Kaskel et al. 2011), and this is accompanied by tubular atrophy and interstitial fibrosis that is thought to result from the continuous exposure of the tubular cells to an excessive load of proteins (Eddy and Giachelli 1995). FSGS is associated with a higher likelihood of steroid resistance and progression to ESRD (Appel 2010). FSGS lesions have been further subdivided

in 5 subtypes according to the Columbia classification proposed in 2004 (D'Agati, Fogo et al. 2004) that is based on the localization of the lesions (urinary pole *vs* glomerular hilus (vascular pole)), the glomerular cellularity and presence of vascular collapse. These variants include: not-otherwise-specified (NOS), tip, peri-hilar, collapsing and cellular (**Figure 6**), and each is associated to a specific clinical prognosis, that is the best for tip lesions and the worst for the collapsing variants (D'Agati, Kaskel et al. 2011).

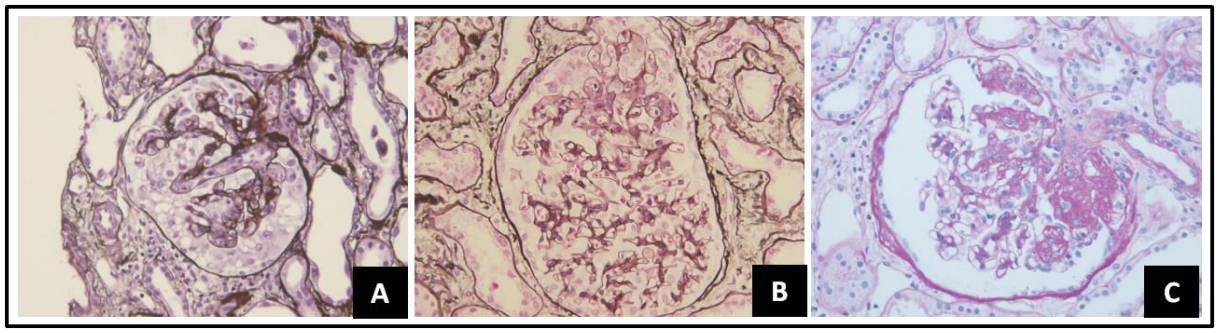

Figure 6. Histologic variants of FSGS. (A) Collapsing variant showing capillary collapse and podocyte hypertrophy **(B)** Tip variant showing a segmental lesion of the glomerular tuft next to the urinary pole **(C)** Peri-hilar variant showing sclerosis and hyalinosis of the vascular pole. Adapted from Lim *et al.* 2016.

Diffuse Mesangial Sclerosis

Typically this lesion presents with mesangial matrix expansion that may be accompanied by podocyte hypertrophy over the capillary tufts (Habib, Gubler et al. 1993). In later stages widening of the GBM, global sclerosis of the glomeruli and tubular atrophy with interstitial fibrosis may supervene. Immuno-fluorescence can show non-specific deposit of IgM, C3 and C1q. By EM the common lesion to all forms of NS, foot process effacement, is observed. DMS is mostly seen in cases of Denys-Drash and Frasier syndromes (caused by *WT1* mutations) Galloway-Mowat syndrome (caused by *WDR73* mutations), Pierson syndrome (caused by *LAMB2* mutations) as well as in cases of isolated SRNS associated to *PLCE1* mutations (Hinkes, Wiggins et al. 2006).

Mechanisms of podocyte injury and glomerulosclerosis

Podocyte detachment, incapacity for cell division and glomerulosclerosis

Research in recent years seems to concede that podocyte depletion is the main initiator of the glomerulosclerosis process. Apoptotic death of podocytes seems to be a quite rare event *in vivo* (Kriz and Lemley 2015), while detaching podocytes have been found in several models of glomerular disease (Kriz, Shirato et al. 2013). The detachment is preceded by foot process effacement, and ultimately leads to the loss of viable podocytes into the urine (Kriz, Shirato et al. 2013). The remaining podocytes theoretically have two possible mechanisms of adaptation in order to cover the denuded GBM at the sites of podocyte loss: either hypertrophy or hyperplasia. However, podocytes are terminally differentiated cells; as such they are unable to proliferate or to respond to the decrease in cell number by cell division (D'Agati, Kaskel et al. 2011). In fact, it has been shown that podocytes forced to exit cell cycle arrest become polyploid and eventually enter a cellular event called mitotic catastrophe (Liapis, Romagnani et al. 2013). The cell division process itself demands complex alterations of the cytoskeleton and cell morphology. In the context of a cell that is exposed to the permanent shear stress of fluid flow across the GFB, these alterations likely also lead to reduced adhesion of the podocyte to the basement membrane and expose the cell to the risk of detachment. In fact binucleated podocytes, thought to result from an incomplete mitosis, have been observed in several types of glomerular disease and show foot process effacement (Nagata, Yamaguchi et al. 1995, Vogelmann, Nelson et al. 2003, Kriz, Shirato et al. 2013, Liapis, Romagnani et al. 2013, Hodgin, Bitzer et al. 2015). In rodent models it has been shown that in response to antibody-complement mediated podocyte injury the levels of cyclin A and cyclin dependent kinase 2 increase (Shankland, Floege et al. 1997), driving these cells to G2/M phases. However, this is limited by a simultaneous increase in cyclin kinase inhibitors such as p21 and p27 (Shankland, Floege et al. 1997), leading to an aborted mitosis. Moreover, the lack of aurora kinase B in mature podocytes also disables the formation of an efficient mitotic spindle (Lasagni, Ballerini et al. 2010). Recently *in vitro* studies on mouse immortalized podocytes grown in non-permissive conditions (and thus leading to differentiation) showed that re-entry in S phase due to stress stimuli induces binucleation and leaves these cells more susceptible to death by a secondary injury (Hagen, Pfister et al. 2016).

Even in normal physiological circumstances there is a continuous loss of podocytes, which can be found in urine of healthy individuals, although at a relatively low level when compared to individuals with glomerular disease. In the adult kidney each glomerulus has approximately 600 podocytes and the normal loss of podocytes has been calculated to be about 1-2 podocytes per glomerulus per year

(Hodgin, Bitzer et al. 2015). In fact, in kidneys from older individuals the density of podocytes is lower than in young kidneys and this is accompanied by a compensatory mechanism of podocyte hypertrophy (Hodgin, Bitzer et al. 2015).

If an inciting injury is strong or prolonged enough to decrease the podocyte number past a threshold level where glomerular survival is no longer possible, glomerular scarring occurs. (Wiggins 2007) **(Figure 7)**. In experimental rodent models where podocytes were genetically modified to become susceptible to specific toxins (i.e. *Diphtheria* toxin or *Pseudomonas* exotoxin), it has been shown that the loss of more than 40% of podocytes leads to glomerulosclerosis with proteinuria and renal failure (Matsusaka, Xin et al. 2005, Wharram, Goyal et al. 2005). Moreover, after the initial lesion, there is a secondary autonomous process that maintains injury and loss of the remnant podocytes (Sato, Wharram et al. 2009, Matsusaka, Sandgren et al. 2011). This could be related to increased shear stress on the remaining podocytes and accumulation of oxidative stress and DNA damage. Interestingly, the ability of podocytes to resist injury may depend on the capacity to resist genotoxic stress. This conclusion came from a study that analyzed why susceptibility to adriamycin-induced glomerulosclerosis occurred only in certain mouse strains. Adriamycin is a cytotoxic drug which has pleiotropic effects, such as introduction of double strand DNA breaks, topoisomerase inhibition, mitochondrial DNA depletion, lipid peroxidation and disruption of the cytoskeleton. Gharavi´s group demonstrated that susceptible strains had a recessive loss of function mutation on *Prkdc* gene that encodes a nuclear DNA double strand break repair protein. Decreased expression of *Prkdc* rendered resistant mice strains susceptible to adriamycin-induced glomerulosclerosis and *in vitro* podocytes more susceptible to adriamycin induced apoptosis and to mitochondrial DNA depletion (Papeta, Zheng et al. 2010).

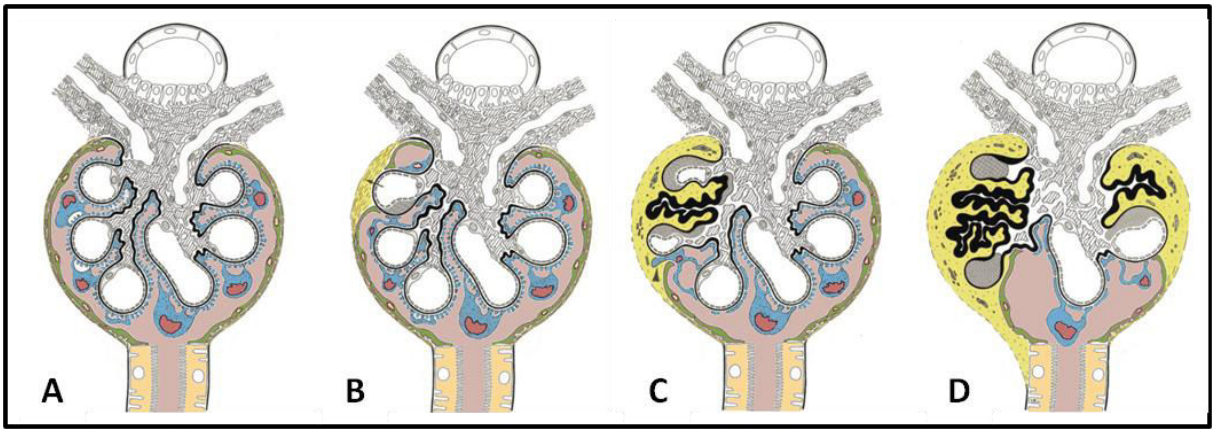

Figure 7. Progression of glomerular diseases. (A) Normal glomerulus; podocytes are shown in blue, parietal epithelial cells in green. The GBM is shown in black **(B)** Initial lesion: Podocyte-denuded capillary attach to Bowman's capsule. **(C)** Progression of the sclerotic process. The adhesion has spread to neighboring capillaries resulting in collapse or in hyalinosis (shown as a thick black line) of the involved capillaries. A paraglomerular space (shown in yellow) containing the sclerotic tuft remnants is formed. **(D)** Late stage of the sclerotic process. A small "intact" tuft still remains. Fibroblasts invade the sclerotic area, resulting in fibrous organization. Adapted from Kriz *et al.* 1998.

Podocyte regeneration by stem cells

In the last two decades, the discovery of renal stem cells has shed some light upon the possibility of podocyte replacement. There are two different types of renal cells that can possibly generate podocytes, the parietal epithelial cells (PECs) and the cells of renin lineage (CoRL). Besides kidney stem cells, there are also several lines of evidence that point to bone marrow stem cells as possible precursors of podocytes.

PECs, a double-edged sword: podocyte regeneration *vs* glomerulosclerosis

PECs line the Bowman's capsule and morphologically resemble squamous epithelial cells. During development, PECs and podocytes have a common mesenchymal cell origin, and then differentiate into two different cell types. Although not much is known about PEC function, they are known to express tight junction molecules (claudin-1, zonula occludens-1, and occludin) and to have a barrier function against the spill of proteins from the primary urine into the interstitial space (Ohse, Chang et al. 2009). Whether PECs are able to regenerate podocytes is a controversial subject. Some PECs express CD24 and CD133 stem cell markers (Ronconi, Sagrinati et al. 2009) and have a known regenerative capacity (Sagrinati, Netti et al. 2006). Interestingly, PECs express differentiation or stem cell markers according to their location on the Bowman's capsule. Those located far from the vascular pole exhibit only stem cell markers and those that are near exhibit only podocyte markers (i.e. nestin, and podocalyxin), while both markers are expressed between the two locations (Ronconi, Sagrinati et al. 2009). Genetic lineage tracing experiments have given non consensual results for the regeneration of podocytes from PECs (Berger, Schulte et al. 2014, Sakamoto, Ueno et al. 2014) (Wanner, Hartleben et al. 2014). In fact, podocyte regeneration seems to depend on the type of glomerular injury and the kidney age. i.e. in juvenile mice where PECs were genetically traced, a subpopulation of these cells was described to migrate along the Bowman's capsule and to express progenitor cell markers and podocyte markers, integrating the population of podocytes resident on the glomerular tuft (Appel, Kershaw et al. 2009). However, in adult mice, spontaneous migration or migration induced by lesion (glomerular hypertrophy by partial nephrectomy) was no longer seen (Berger, Schulte et al. 2014). In human kidneys analogous observations were made, among the PECs, podocyte markers have scattered expression until 2 years of age, but not on PECs from 7 year old individuals (Berger, Schulte et al. 2014). In another lineage tracing experiment, no regeneration occurred in ageing mice or after nephron reduction, however regeneration did occur in response to acute podocyte loss induced in the diphtheria toxin model (Wanner, Hartleben et al. 2014), and this

finding was replicated in another mouse model of acute podocyte depletion (Eng, Sunseri et al. 2015).

If the regeneration of podocytes from PECs is still a controversial subject, several lines of evidence have firmly established that an excessive proliferative response by PECs after glomerular injury can lead to the formation of glomerular lesions such as cellular or fibrous crescents and collapsing lesions in FSGS (Lasagni and Romagnani 2010). During injury, PECs can undergo phenotypic changes that have been described by the general term of "activation" (Shankland, Smeets et al. 2014). These include changes in morphology (larger cytoplasm and nucleus) as well as increased migration, proliferation and synthesis of extra-cellular matrix, and *de novo* expression of marker proteins, such as CD44 (a cell surface adhesion receptor) (Shankland, Smeets et al. 2014). In several mouse models of glomerular injury using genetic lineage tracing of PECs, and in human transplant biopsies, it has been shown that activated PECs can migrate to the glomerular tuft through adhesion sites formed between the tuft and the Bowman capsule, and produce extracellular matrix, contributing to the development of sclerosis (Appel, Kershaw et al. 2009, Smeets, Kuppe et al. 2011, Smeets, Stucker et al. 2014). Interestingly the presence of activated PECs may distinguish between the sclerosing lesions of FSGS and the absence of fibrosis in MCD, with the former showing invasion of the glomerular tuft by CD44 positive PECs (Smeets, Stucker et al. 2014). A very detailed study on human biopsies of several types of glomerular disease using multiple markers for PECs, activated PECs and podocytes has clearly defined that the earliest lesion is the formation of a cellular bridge between the Bowman's capsule and the glomerular tuft, formed by PECs that deposit matrix in the tuft, which is followed by the migration of PECs onto the tuft and the formation of fibrotic adhesions or early sclerotic lesions, and finally the formation of advanced sclerotic lesions, where a larger part of the tuft is covered by PECs that continue to deposit extracellular matrix (Kuppe, Grone et al. 2015) (**Figure 8**).

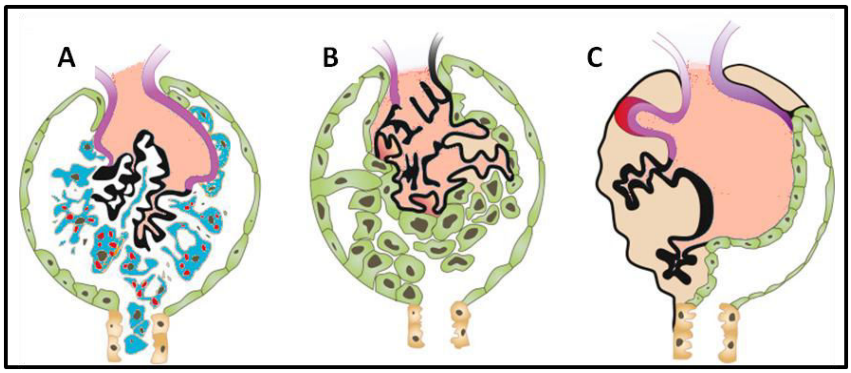

Figure 8. Parietal epithelial cells participate in the progression of glomerulosclerosis. (A) Severe injury leading to fulminant loss of podocytes **(B)** Activation and migration of PECs to the glomerular tuft through synechiae formed between the denuded GBM and the Bowman's capsule **(C)** Late stages of glomerulosclerosis showing a monolayer epithelium of PECs covering the glomerular basement membrane. PECs further synthesize extracellular matrix that leads to complete sclerosis. Adapted from Nagata *et al.* 2016.

A recent study has shown that for effective podocyte regeneration in response to a glomerular injury, PECs activation has to be followed by differentiation and expression of podocyte markers, otherwise, the maintenance of activated PECs leads to glomerulosclerosis. In a mouse model of FSGS with genetic lineage tracing of PECs, these authors demonstrated that if activated PECs successfully differentiated into podocytes the disease remitted, while glomerulosclerosis occurred if these cells were not able to differentiate (Lasagni, Angelotti et al. 2015). Promisingly, enhancing the activity of endogenous retinoic acid, that controls differentiation in podocytes, with a glycogen synthase kinase3 inhibitor significantly increased the trans-differentiation of PECs into podocytes and increased disease remission (Lasagni, Angelotti et al. 2015). Currently several other molecules that target molecular pathways associated with PEC proliferation, activation and extracellular matrix production are being further studied in animal models (Miesen, Steenbergen et al. 2017).

CoRL and bone marrow derived stem cells

CoRL are vascular smooth cells that under normal conditions produce renin and are localized to the juxtaglomerular compartment, between afferent and efferent arterioles. These cells have been found to be able to lose their characteristic endocrine and contractile functions and differentiate into several adult cell types, including mesangial cells (Thoma 2014, Castellanos-Rivera, Pentz et al. 2015), pericytes (Castellanos-Rivera, Pentz et al. 2015, Pippin, Kaverina et al. 2015), vascular smooth cells (Castellanos-Rivera, Pentz et al. 2015, Pippin, Kaverina et al. 2015) , erythropoietin-producing cells, hematopoietic immune-like cells (Castellanos Rivera, Monteagudo et al. 2011), PECs and podocytes (Shankland, Pippin et al. 2014). In a rodent model of acute podocyte depletion, cell fate tracing of CoRL and *in vivo* time lapse imaging showed that these cells populate the glomeruli and differentiate in PECs and podocytes (Kaverina, Kadoya et al. 2017). The same has been shown in models of chronic podocyte depletion (Pippin, Kaverina et al. 2015).

Other sources of podocyte progenitors have been described such as the bone marrow-derived stem cell source, as evidenced by the presence of 7% of podocytes derived from a male recipient on a female kidney allograft (Becker, Hoerning et al. 2007). Moreover, injection of bone marrow-derived stem cells ameliorates adriamycin induced FSGS lesions (Uchida, Kushida et al. 2017). However, discrepant results have been found in other studies (Meyer-Schwesinger, Lange et al. 2011), arguing for the need of more intensive investigation in this field before considering bone marrow-derived stem cells as definite podocyte progenitors.

The podocyte slit diaphragm, lipid rafts and actin cytoskeleton

The slit diaphragm

The slit diaphragm was first visualized by electron microscopy (EM) in 1955 (Yamada 1955) and it was only in 1974 that with better EM techniques the classic view of the slit diaphragm as a zipper-like structure was described (Rodewald and Karnovsky 1974, Karnovsky and Ryan 1975). In this model the slit diaphragm molecules from neighboring foot processes are thought to cross the extracellular space and are arranged in a dense midline, corresponding to the overlapping region of their extracellular domains (**Figure 9**). The pores between these strands, of about 12 nm, would be small enough to prevent the passage of albumin or higher molecular weight proteins. In the same year, the loss of the slit diaphragms and the process of foot process effacement in response to podocyte injury were also described (Robson, Giangiacomo et al. 1974). These findings provided a strong link between slit diaphragm loss, foot process effacement and proteinuric glomerular diseases.

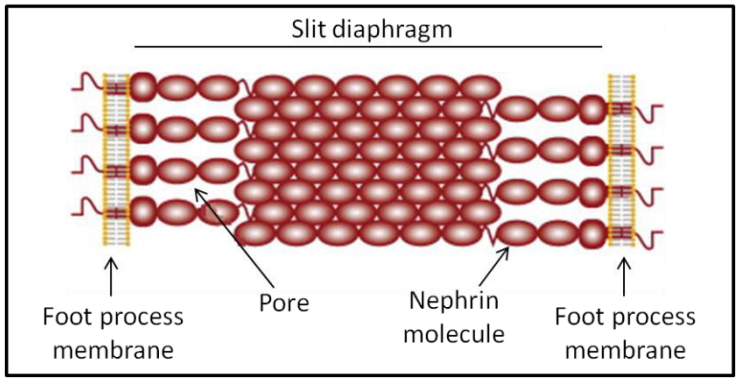

Figure 9. The slit diaphragm zipper-like structure. Nephrin molecules from neighboring foot processes interact at the center of the slit, forming a zipper-like structure with pores on both sides of the central density. Adapted from Patrakka *et al.* 2007.

In recent decades genetic studies have contributed to a better understanding of the molecular composition of the slit diaphragm. In 1998, through a positional cloning approach, researchers identified the first gene known to be mutated in congenital cases of NS, *NPHS1*, encoding for the transmembrane Immunoglobulin superfamily protein nephrin (Kestila, Lenkkeri et al. 1998). In subsequent years nephrin was identified as the backbone molecule of the slit diaphragm filter (Ruotsalainen, Ljungberg et al. 1999, Wartiovaara, Ofverstedt et al. 2004). This was followed by the identification of *NPHS2* (Boute, Gribouval et al. 2000), encoding podocin, a slit diaphragm associated protein. This is the most frequently mutated gene in familial cases of NS (Bouchireb, Boyer et al. 2014). In the following years other critical components of the slit diaphragm such as the Immunoglobulin superfamily nephrin like protein-1 (Neph1), as well as proteins linking the slit diaphragm to the actin cytoskeleton, were discovered.

Very recently, development of different fixation and EM techniques, such as cryo-electrontomography and focused ion beam-scanning electron microscopy (FIB-SEM), permitted a review of the classical zipper-like structure of the slit diaphragm (Burghardt, Hochapfel et al. 2015, Grahammer, Wigge et al. 2016). Instead of a monolayer of nephrin and Neph1 that establish homo or heterophilic interactions, the slit diaphragm seems to have superposed layers of these transmembrane proteins. Both molecules seem to completely span the interpodocytary space and do not form homo or heteromeric interactions between them, contrary to the previous belief (Burghardt, Hochapfel et al. 2015, Grahammer, Wigge et al. 2016). Neph1 forms the bottom layers, close to the GBM, spanning a distance of 23 nm, nephrin molecules on the other hand form the upper layers and span a distance of around 45 nm. Both molecules are separated by a distance of 7 nm. **(Figure 10)**. The absence of a well-defined pore structure with certain dimensions raises the question on how this structure contributes to the permselectivity of the GFB, a question that will most certainly be addressed in the coming years, and that should take into account other slit diaphragm proteins.

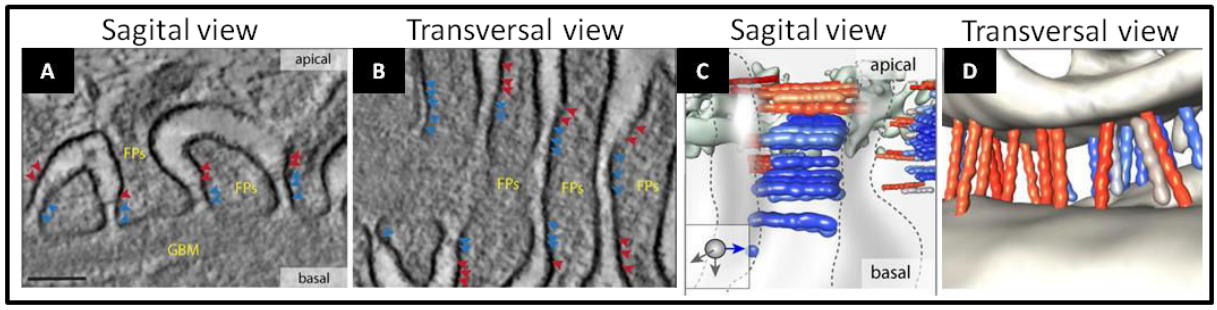

Figure 10. New EM techniques suggest a novel slit diaphragm structure. The slit diaphragm is suggested to be a multilayered, bipartite protein scaffold. **(A)** High-pressure freezing and plastic electron tomography (PET) show several strands crossing the space between different FP. The narrower end of the slit is toward the GBM, while the broader end is towards the Bowman's space (blue arrowheads mark shorter strands and red arrowheads correspond to longer strands). **(B)** Transversal view showing a quasiperiodical distribution of the slit layers **(C)** 3D reconstitution showing foot process bridging strands color-coded depending on their length (red 40 nm, attributed to nephrin, blue 25 nm, attributed to Neph1) **(D)** Transversal view. Adapted from Grahammer *et al.* 2016.

The slit diaphragm is a specialized type of cell junction that exhibits characteristics of both tight junctions, expressing proteins such as ZO-1, occludin and cingullin (Schnabel, Anderson et al. 1990, Fukasawa, Bornheimer et al. 2009), and of adherens junctions, expressing P-cadherin or α, β, γ catenin proteins (Reiser, Kriz et al. 2000, Lehtonen, Lehtonen et al. 2004). During podocyte development the slit diaphragm is not the first type of cellular junction to be formed. The first cellular junction is an apical tight junction (at the S stage) when podocyte precursors form a cobblestone-like epithelium (Ruotsalainen, Patrakka et al. 2000, Ichimura, Kakuta et al. 2017). Later in development these junctions migrate to the more baso-lateral aspect of the podocyte membrane (Ichimura, Kakuta et al. 2017) and become enriched with adherens junction proteins, nephrin and

other proteins from the Immunoglobulin superfamily (Goto, Yaoita et al. 1998). During this process the podocyte junctions become wider, suggesting that nephrin proteins serve a repulsive action to maintain open pores of a certain diameter. Interestingly, in disease conditions a reverse phenomenon is seen, where slit diaphragms are replaced by tight junctions and podocytes retract their foot processes and regain the typical epithelial cobblestone appearance (Kurihara, Anderson et al. 1992, Kriz, Shirato et al. 2013).

Besides the well-known filter function, slit diaphragm proteins also serve as a linker to the podocyte actin cytoskeleton and form a signaling platform that allows integration of extracellular signals, such as shear stress and mechanical forces, paracrine and autocrine signals, and intracellular signals (Grahammer, Schell et al. 2013). These functions contribute to plasticity of foot processes and to podocyte homeostasis.

Components of the slit diaphragm and activation of cellular signaling

The slit diaphragm proteins are linked to a large complex composed of multiple proteins that facilitate coupling with the underlying actin cytoskeleton and signal transduction **(Figure 11)**. Underlining the complexity of this structure, proteins associated to the slit diaphragm continue to be identified in genetic studies of Mendelian SRNS. This is the case of Crumbs homolog 2 (CRB2), shown to colocalize with slit diaphragm proteins and be required for nephrin trafficking (Ebarasi, Ashraf et al. 2015), and more recently the membrane-associated guanylate kinase 2 (MAGI2) that interacts with nephrin and also regulates slit diaphragm dynamics (Bierzynska, Soderquest et al. 2017). On this section I will briefly describe some of the most well known slit diaphragm-associated proteins, most of them encoded by genes mutated in SRNS.

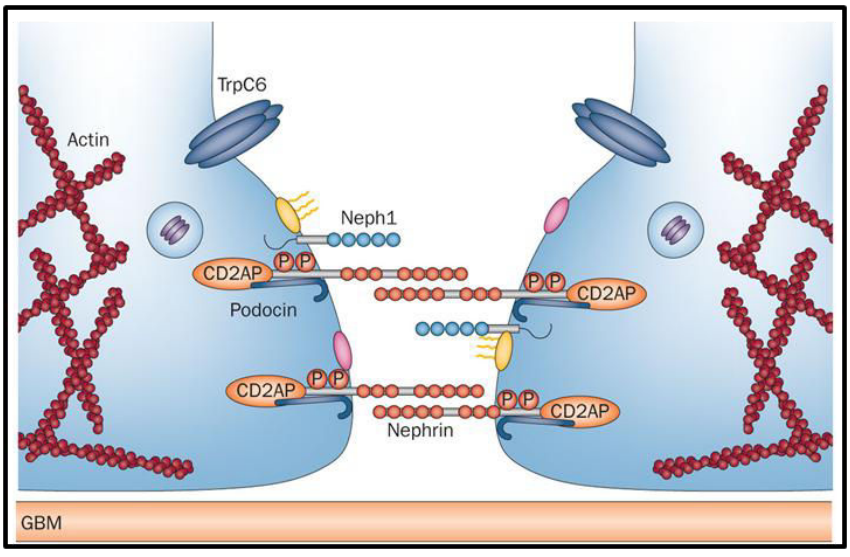

Figure 11. Main molecular components of the slit diaphragm. Some of the most well known slit diaphragm proteins as well as their spatial organization are depicted in the figure. The slit diaphragm and its associated proteins form a signaling hub that control actin cytoskeleton and intracellular signaling. Adapted from Grahammer *et al.* 2013.

Nephrin

Nephrin is a type 1 transmembrane protein. The cytoplasmic 150 amino acid domain has several tyrosine-based motifs. It is followed by a transmembrane domain and an extracellular portion of around 44 nm that contains a fibronectin type III domain and eight C2-type immunoglobulin domains. The tyrosine motifs in the intracellular portion of the protein can bind and be phosphorylated by tyrosine protein kinases such as Fyn (Verma, Wharram et al. 2003). Interestingly in mouse models, *Fyn* KO leads to decreased nephrin phosphorylation and disrupted foot processes (Verma, Wharram et al. 2003). The phosphorylated tyrosine residues serve as binding sites for the Nck adaptor protein, known to activate regulators of the actin cytoskeleton such as the neural Wiskott-Aldrich-syndrome (N-WASP), and p21 activated kinase (PAK) (Jones, Blasutig et al. 2006) (Verma, Kovari et al. 2006, Zhu, Attias et al. 2010). Interestingly the small GTPases RAC1 and Cdc42 can interact with N-WASP, P21 and other nephrin partners (Brandt and Grosse 2007, Harita, Kurihara et al. 2009). Phosphorylated nephrin also binds the phosphoinositide 3-kinase (PI3K), then stimulating AKT, which controls cell growth and survival as well as actin remodeling (Huber, Hartleben et al. 2003, Canaud, Bienaime et al. 2013).

Nephrin can also interact with Iq motif containing GTPase-activating protein (IQGAP) that is able to recruit the actin related protein (Arp) 2/3 complex (Liu, Liang et al. 2015). Other molecules that are known to lead to FSGS in murine models or in humans, like CD2AP and PTPRO, are also known to interact with nephrin (Li, Lemay et al. 2004, Aoudjit, Jiang et al. 2011, Ozaltin, Ibsirlioglu et al. 2011). All these findings link nephrin signaling to the underlying actin cytoskeleton and to survival signals that may modulate podocyte morphology and homeostasis.

Neph1

Like nephrin, Neph1 protein belongs to the Ig superfamily proteins, sharing a similar structure. In comparison to nephrin its extracellular region has only 5 C2-type immunoglobulin domains and no fibronectin domain, spanning only 20 nm. Neph1 has an important role in maintaining a healthy GFB as shown by the development of congenital NS in constitutive *Neph1* KO mice (Donoviel, Freed et al. 2001, Grahammer, Wigge et al. 2016), which was not the case for the KO of its paralogs *Neph-2* nor *Neph-3*, that do not seem to be expressed in the kidney (Grahammer, Wigge et al. 2016). Through its intracellular C-terminal domain Neph1 also establishes several important interactions with scaffolding molecules, such as ZO-1 (Mallik, Arif et al. 2012), and polarity regulators such as the

aPKC/Par3/Par6 complex (Hartleben, Schweizer et al. 2008). Neph1 and nephrin directly interact (Garg, Verma et al. 2007). Similar to nephrin, Neph 1 phosphorylation also modulates interaction with other partners, such as Grb2, and plays a major role in regulating the actin cytoskeleton (Garg, Verma et al. 2007).

Podocin

Podocin is a 42KDa protein from the stomatin family. It is anchored to the membrane by its hydrophobic central part and usually bends in a hairpin configuration, with both C-terminus and N-terminus facing the cytosol (Roselli, Gribouval et al. 2002). It has podocyte-specific expression and localizes to lipid rafts (Roselli, Gribouval et al. 2002). As further discussed (see "Podocyte Lipid Rafts" chapter) it is involved in the recruitment of cholesterol through its prohibitin-like (PHB) domain as well as other protein partners. These partners include nephrin, CD2AP and TRPC6 (Schwarz, Simons et al. 2001, Huber, Simons et al. 2003, Huber, Schermer et al. 2007). All assemble in a macromolecular complex that forms the signaling platform of the slit diaphragm.

CD2AP

Although CD2AP protein was first studied because of its ability to stabilize the interactions between T-cells and antigen-presenting cells, the main phenotype of the *Cd2ap* KO mouse turned-out to be congenital NS and early renal failure (Shih, Li et al. 1999). CD2AP is a scaffold protein known to interact with F-actin, nephrin and podocin (Schwarz, Simons et al. 2001). It has three SH3 domains and a proline-rich motif that binds to F actin capping protein (CAPZ), and a C-terminal coiled coil domain (Edwards, Zwolak et al. 2014). The SH3 domains and the proline rich motif allow CD2AP to establish interactions both with slit diaphragm partners and with regulatory proteins of the cytoskeleton such as CAPZ synaptopodin and Rac1 (Faul, Asanuma et al. 2007, van Duijn, Anthony et al. 2010). However, to date autosomal recessive mutations in the *CD2AP* gene have been described in only one family (Lowik, Groenen et al. 2007).

TRPC6

Transient receptor potential channel, subfamily C, member 6 (TRPC6) is a non-selective cation channel of the TRPC subfamily, that drives cell depolarization. TRPC6 is sensitive to diacylglycerol

(DAG) and allows calcium entry into the cell (Hofmann, Obukhov et al. 1999) that in turn modulates secondary cascades, resulting in integration or amplification of cell signals (Dryer and Reiser 2010). In podocytes TRPC6 is known to interact with nephrin and podocin, also localizing to lipid rafts (Huber, Schermer et al. 2006, Lei, Lu et al. 2014) and forms a tetrameric ion channel that may respond to changes in pressure or fluid flow. Increase in intracellular calcium through TRPC6 has been shown to stimulate RhoA activity thereby modulating the actin cytoskeleton and the foot process shape (Moller, Flesche et al. 2009, Jiang, Ding et al. 2011).

TRPC6 was found to be mutated in autosomal dominant SRNS (Reiser, Polu et al. 2005, Winn, Conlon et al. 2005). Most of the mutations are gain-of-function mutations that induce a continuous activation of TRPC6 and positive calcium influx into the cell, leading to apoptosis *in vitro* (Reiser, Polu et al. 2005, Winn, Conlon et al. 2005). Consistent with a gain-of-function mechanism, *Trpc6* KO mice have no overt renal phenotype however podocyte-specific overexpression of TRPC6 leads to FSGS (Krall, Canales et al. 2010, Li, Ding et al. 2017). However, recently, dominant negative mutations of TRPC6 have also been described (Riehle, Buscher et al. 2016), suggesting that both TRPC6 hyper and hypo-activation can lead to disease in humans. Interestingly *TRPC6* mutations have variable penetrance and healthy carriers have been observed (Sun, Ng et al. 2015). This could be explained by differences in genetic background (Sun, Ng et al. 2015).

FAT1

FAT1 is a type 1 transmembrane protein that belongs to the cadherin family, harboring 33 cadherin repeats, a laminin G domain, and 2 EGF domains in its extracellular region. FAT1 has been shown to be crucial for the podocyte slit diaphragm formation in mouse models (Ciani, Patel et al. 2003) and co-localizes with other slit diaphragm proteins (Inoue, Yaoita et al. 2001). *In vitro* FAT1 has been shown to participate in Ena/VASP-dependent regulation of cytoskeleton dynamics (Moeller, Soofi et al. 2004) and *Fat1* KO mice exhibit effacement of foot processes and defects in eye and central nervous system development (Ciani, Patel et al. 2003). Recently, mutations in *FAT1* have been described in four families and cause a glomerular disease with either diffuse mesangial sclerosis or minimal change disease associated with tubular ectasia. Moreover there is facultative neurological involvement and patients may develop different kinds of tumors (Gee, Sadowski et al. 2016).

Podocyte lipid rafts

The plasma membrane is a fluid lipid bilayer with micro-domains of increased rigidity due to the enrichment of cholesterol, glycolipids and sphingolipids. These structures, called the lipid rafts, are highly dynamic and regulate membrane fluidity, membrane protein trafficking and protein clustering, facilitating the initiation of downstream signaling (Simons and Ikonen 1997). Many receptors of the tyrosine kinase family and their effectors are enriched in these micro-domains and this seems to be critical for the type and duration of the downstream signal and the cellular outcomes (Pike 2003). Interestingly, in podocytes some slit diaphragm proteins are known to localize to lipid raft domains and podocin plays a central role in this compartmentalization. In fact podocin PHB domain (a 150 amino acid domain similar to the one found in the mitochondrial protein prohibitin) is able to bind and recruit cholesterol. Along with cholesterol binding, podocin also binds to TRPC6 channels as well as other slit diaphragm proteins, allowing their recruitment to the podocyte lipid raft (Schwarz, Simons et al. 2001, Huber, Schermer et al. 2006). Moreover, the regulation of the activity of TRPC6 channels is dependent on the interaction with podocin and cholesterol (Huber, Schermer et al. 2006, Huber, Schermer et al. 2007). This creates protein-cholesterol super-complexes in the plasma membrane that are critical to mechanosensation and transduction of mechanical stimuli into ion fluxes to the intracellular space (Huber, Schermer et al. 2007).This macromolecular structure is highly similar to the *C. elegans* mechano-sensory apparatus of touch receptor neurons, where the podocin homolog Mec-2 organizes a cholesterol-rich platform that assembles ion channels, transmembrane proteins and linkers to the cytoskeleton (Huber, Schermer et al. 2007).

The podocyte actin cytoskeleton and its regulation

Regulation of the actin cytoskeleton is critical to maintain the complex podocyte architecture. Factors that promote an overly rigid cytoskeleton or one that is too dynamic may perturb podocyte morphology and function. In accordance, several proteins that directly or indirectly participate in actin cytoskeleton dynamics are known to be mutated in familial forms of SRNS (Perico, Conti et al. 2016). In podocytes the cell body and primary and secondary processes are mainly composed of intermediate filaments and microtubules; while foot processes have long actin bundles that extend longitudinally and a subcortical actin ring (Faul, Asanuma et al. 2007). Besides the cyto-architectural function of actin filaments, these structures can also serve as trackers for the transport of several molecules and play a major role in cell migration and adhesion. Recently, the importance of the microtubule cytoskeleton in podocyte biology was highlighted by our laboratory, as mutations in

WDR73 and *TTC21B* were found in hereditary NS and shown to disrupt the microtubule organization in podocytes (Colin, Huynh Cong et al. 2014, Huynh Cong, Bizet et al. 2014). This chapter describes the main players on podocyte actin cytoskeleton, with special relevance for the ones found to be associated to SRNS in humans.

The small RhoGTPases RhoA, Rac1 and Cdc42

The mechanism for actin filament assembly and disassembly is highly regulated by several protein families. Among them, the family of small RhoGTPases, including the RhoA, Rac1, and Cdc42 proteins, plays a very important role. These proteins are active when binding to GTP and inactive when bound to GDP. Their activation status is in turn regulated by three different proteins: At the level of the plasma membrane, the guanine nucleotide exchange factors (GEFs) exchange a GDP molecule for a GTP molecule, activating the RhoGTPases. The GTP activating factors (GAP) inactivate the RhoGTPases by hydrolysis of the GTP molecule. The guanine dissociation inhibitors (GDIs) prevent the activation of the RhoGTPases by binding them in the cytosolic compartment, not allowing their activation by GEFs at the plasma membrane. RhoA, Rac1 and Cdc42 exert their actions in a tightly regulated manner. In a simplified way, RhoA is known to signal through its effectors formins and ROCK, promoting the formation of contractile actomyosin. Although Rac1 and Cdc42 can also trigger formin activation, they are mostly known to promote Arp2/3-dependent branched actin structures, through WAVE and N-WASP, respectively (Arnold, Stephenson et al. 2017).

Interestingly, each of these RhoGTPases has been shown to play a role in podocyte function. Synaptopodin, a protein well known for its main role on the organization of the podocyte actin cytoskeleton, regulates the activation of RhoA and Cdc42 (Asanuma, Yanagida-Asanuma et al. 2006, Yanagida-Asanuma, Asanuma et al. 2007).

RhoA activation has been linked to worsen glomerular lesions in some rodent models of glomerular disease, such as diabetic nephropathy (Komers, Oyama et al. 2011) or PAN-induced nephropathy in rats (Shibata, Nagase et al. 2006). In addition mutations in *KANK2*, encoding KN motif and ankyrin repeat domain-containing protein 2, were found in humans with NS and shown to lead to RhoA activation *in vitro* (Gee, Zhang et al. 2015).

Rac1 deregulation has also been linked to podocyte injury. An inactivating mutation in *ARHGAP24*, encoding the Rho-GTPase-activating protein 24, has been found in one family with FSGS and *in vitro* silencing of ARHGAP24 in podocytes increased activation of Rac1 and Cdc42 (Akilesh, Suleiman et al. 2011). Moreover, FSGS due to biallelic inactivating mutations in *ARHGDIA*, encoding the Rho GDP-

dissociation inhibitor 1, a GDI that prevents the activation of RhoGTPases, has been described by several groups (Gee, Saisawat et al. 2013, Gupta, Baldwin et al. 2013). *In vitro ARHGDIA* mutations induce Rac1 activation (Gee, Saisawat et al. 2013, Gupta, Baldwin et al. 2013, Auguste, Maier et al. 2016) and pharmacological inhibition of Rac1 activity in *Arhgdia* KO mice or in morpholino loss-of-function zebrafish models, improved kidney lesions (Shibata, Nagase et al. 2008, Gee, Saisawat et al. 2013). In a transgenic mouse line where constitutively active Rac1 was selectively expressed in podocytes, proteinuria and foot process effacement developed (Yu, Suleiman et al. 2013). However while podocyte specific deletion of *Rac1* in mice protected against the acute podocyte lesion induced by protamine, in a long term model of hypertensive glomerular disease it actually aggravated proteinuria and glomerulosclerosis (Blattner, Hodgin et al. 2013). These studies suggest that Rac1 activity can have either beneficial or deleterious effects depending on the context and the activation level.

Cdc42 has more recently been shown to be important for podocyte homeostasis. In fact, although neither *Rac1* nor *RhoA* podocyte specific KO in mice led to foot process effacement or proteinuria, *Cdc42* KO led to collapsing FSGS and induced abnormal junctional complexes in podocytes (Scott, Hawley et al. 2012).

INF2

Mutations in *INF2*, encoding the inverted formin 2, cause an autosomal dominant form of FSGS in early adulthood (Brown, Schlondorff et al. 2010) potentially associated with a Charcot-Marie-Tooth neuropathy (Boyer, Nevo et al. 2011). INF2 is an effector of Cdc42 and is able to promote linear actin polymerization through the so-called formin homology domains (FH1 and FH2). INF2 can also promote the depolymerization of actin microfilaments (Chhabra and Higgs 2006). INF2 belongs to the group of "diaphanous formins" that have the particularity of auto-inhibition *via* interaction between an N-terminal diaphanous inhibitory domain (DID) and a C-terminal diaphanous auto-regulatory domain (DAD) (Chhabra and Higgs 2006). Interestingly all the mutations leading to FSGS are located on the DID domain. INF2 DID domain can also bind to the DAD domain of the “mammalian diaphanous related formin” mDia and inhibit its Rho-activated actin polymerization, lamellipodial dynamics and trafficking of slit diaphragm proteins from the ER to the plasma membrane (Sun, Schlondorff et al. 2013). Interestingly the mutations found in the DID domain perturb the INF2 mediated inhibition of Rho/mDia and result in disturbance of actin dynamics (Sun, Schlondorff et al. 2011, Sun, Schlondorff et al. 2013). Interestingly, INF2 can also interact with the slit diaphragm associated protein IQGAP1 (Bartolini, Andres-Delgado et al. 2016).

α-actinin-4

The α-actinin proteins crosslink actin filaments and act as scaffolds for the assembly of larger protein complexes. These proteins have an actin binding domain, a rod domain, responsible for the crosslinking activity of actin filaments, and a calmodulin-like domain that regulates the actin crosslinking activity through calcium binding. Mutations in *ACTN4*, encoding α-actinin-4, lead to autosomal dominant FSGS (Kaplan, Kim et al. 2000) that manifests in adulthood. The mutations are localized to the actin binding domain and lead to an increased affinity for actin that becomes independent of calcium regulation (Weins, Schlondorff et al. 2007). This leads to the formation of α-actinin-4/F-actin aggregates as observed in cell culture models (Weins, Schlondorff et al. 2007). Transgenic mice expressing *Actn4* mutations develop a kidney disease similar to that found in humans (Michaud, Lemieux et al. 2003, Henderson, Al-Waheeb et al. 2008). Interestingly, *Actn4* constitutive KO mice develop podocyte foot process effacement and glomerulosclerosis, but similar to humans no other organ is affected (Kos, Le et al. 2003). This suggests that recessive loss-of-function mutations in *ACTN4* could be potentially found in humans.

Myosin 1E

MYO1E, encoding myosin 1E, has been found to be mutated in recessive forms of SRNS (Mele, Iatropoulos et al. 2011, Sanna-Cherchi, Burgess et al. 2011). It is a ubiquitously expressed non-muscle class I myosin. These proteins have a single motor domain, a neck domain that binds to calmodulin light chains, and a cargo binding tail domain. Moreover, in podocytes non-muscle myosins are known to form a complex with actin and utrophin that attaches to the basement membrane through α and β dystroglycans (Faul, Asanuma et al. 2007, Nabet, Tsai et al. 2009). *Myo1e* KO mice develop glomerular disease and proteinuria as well as GBM widening (Krendel, Kim et al. 2009) and *in vitro* silencing of myosin 1E in conditionally immortalized podocytes leads to actin cytoskeleton rearrangement and changes in migration, proliferation and decreased adhesion to basement membrane components (Mao, Wang et al. 2013). Moreover, silencing of myosin 1E also disrupts endocytosis (Mao, Wang et al. 2013), which can be explained by its interaction with the endocytic proteins dynamin and synaptojanin (Krendel, Osterweil et al. 2007).

Interestingly, mutations in another myosin, the non-muscle myosin IIa heavy chain, encoded by *MYH9*, cause an autosomal dominant disease of which the main features are

macrothrombocytopenia and leukocyte inclusions, and in a few cases a glomerulopathy (Singh, Nainani et al. 2009).

Anillin

ANLN was recently found to be mutated in two families with autosomal dominant FSGS (Gbadegesin, Hall et al. 2014). Anillin is an F actin binding protein that is known to be important for cell division (Watanabe, Okawa et al. 2010) and cell growth (Suzuki, Daigo et al. 2005). Suggesting its importance for the maintenance of the slit-diaphragm structure in podocytes, in several other types of epithelial cells anillin plays a main role on the maintenance of an apical actomyosin belt and on the regulation of cell-cell junctions, through the control of Rho-GTP and adducin-γ distribution at cell-cell contacts (Reyes, Jin et al. 2014, Wang, Chadha et al. 2015). In podocytes, it is known to interact with key proteins for the actin cytoskeleton, such as RhoA, CD2AP and mDIA2 (Monzo, Gauthier et al. 2005, Watanabe, Okawa et al. 2010, Reyes, Jin et al. 2014). Interestingly, the identified human mutations decrease the interaction with its binding partner CD2AP and overexpression of mutant anillin in immortalized podocytes leads to increased podocyte motility, compared to the WT (Gbadegesin, Hall et al. 2014).

Genetics of steroid-resistant nephrotic syndrome

To date, mutations in more than 40 genes have been identified as a cause of Mendelian SRNS in humans, defining SRNS as a disease with high genetic heterogeneity. These genes encode proteins essential for podocyte actin cytoskeleton and slit diaphragm associated proteins (as reviewed in the previous chapter), but also genes crucial for the microtubule cytoskeleton organisation, adhesion of podocytes to the underlying basement membrane, proteins related to cell polarity, transcription factors, nuclear envelop proteins, and genes essential for mitochondrial or even lysosomal function. All these are detailed in **Table 1**.

In a recent study where 27 known genes for SRNS were tested for the presence of mutations in 1.783 unrelated families worldwide (2016 individuals), a monogenic cause was detected in roughly 30% of the cases with disease onset before 25 years of age (Sadowski, Lovric et al. 2015). Overall the *NPHS2* gene was the most frequently mutated gene between 1 and 18 years of age (Sadowski, Lovric et al. 2015). As described in previous studies (Hinkes, Mucha et al. 2007, Santin, Bullich et al. 2011, McCarthy, Bierzynska et al. 2013), successful identification of the underlying causal mutation was higher when SRNS manifested in the first 3 months of life (≈70%) and gradually decreased with increasing age, being only 11% between 13 and 18 years old (Sadowski, Lovric et al. 2015). These findings support the concept that later in life some SRNS cases may be multifactorial in nature, with multiple genetic variants influencing susceptibility to the disease in response to noxious environmental factors.

The most frequently mutated genes in congenital and infantile NS are *NPHS1, NPHS2, WT1, LAMB2* and *PLCE1*, and their mutations are of autosomal recessive transmission (Sadowski, Lovric et al. 2015). Autosomal dominant mutations in *INF2* and *TRPC6* are more frequent in young adults and have incomplete penetrance and variable expressivity (Brown, Pollak et al. 2014, Sadowski, Lovric et al. 2015). The fact that AD diseases present later in life and have a more variable phenotype is not restricted to SRNS or kidney diseases, and likely depends on other genetic as well as environmental factors.

Table 1. Genes implicated in primary GFB defects leading to SRNS

Gene	Protein	Mode of inheritance	Clinical phenotype	Reference
Encoding proteins associated to the SD				
NPHS1	Nephrin	AR	CoNS and infantile SRNS	(Kestila, Lenkkeri et al. 1998)
NPHS2	Podocin	AR	Infantile and childhood SRNS	(Boute, Gribouval et al. 2000)
CD2AP	CD2-associated protein	AR	Infantile SRNS	(Kim, Wu et al. 2003)
TRPC6	Short transient receptor potential channel 6	AD	Juvenile and adult-onset FSGS	(Winn, Conlon et al. 2005)
FAT1	FAT atypical cadherin 1	AR	Childhood SRNS	(Gee, Sadowski et al. 2016)
MAGI2	Membrane-Associated Guanylate Kinase Inverted 2	AR	CoNS, childhood-onset SRNS	(Bierzynska, Soderquest et al. 2017)
CRB2	Crumbs homolog 2	AR	Early-onset SRNS	(Ebarasi, Ashraf et al. 2015) (Slavotinek, Kaylor et al. 2015)
Encoding proteins related to the cytoskeleton				
ACTN4	α-actinin-4	AD	Juvenile and adult-onset FSGS	(Kaplan, Kim et al. 2000)
INF2	Inverted formin-2	AD	Juvenile and adult-onset FSGS	(Brown, Schlondorff et al. 2010)
MYO1E	Unconventional myosin IE	AR	FSGS	(Mele, Iatropoulos et al. 2011)
MYH9	Myosin, heavy chain 9	AD	FSGS Macrothrombocytopenia with sensorineural deafness, Epstein syndrome, Sebastian syndrome, Fechtner syndrome	(Ghiggeri, Caridi et al. 2003)
ARHGAP24	Rho-GTPase-activating protein 24	AD	Infantile and juvenile FSGS	(Akilesh, Suleiman et al. 2011)
ARHGDIA	Rho GDP-dissociation inhibitor 1	AR	CoNS	(Gupta, Baldwin et al. 2013)
ANLN	Anillin	AD	Adult onset FSGS	(Gbadegesin, Hall et al. 2014)
KANK1/2/4	Kidney ankyrin repeat-containing protein 1/2/4	AR	Childhood SSNS/SRNS	(Gee, Zhang et al. 2015)
TTC21B	Tetratricopeptide repeat domain 21B	AR	Nephronophthisis , FSGS	(Huynh Cong, Bizet et al. 2014)
WDR73	WD repeat domain 73	AR	Galloway-Mowat syndrome, SRNS/FSGS	(Colin, Huynh Cong et al. 2014)
Encoding nuclear proteins				
WT1	Wilms tumour protein	AD	Denys–Drash syndrome, Frasier syndrome or isolated SRNS	(Pelletier, Bruening et al. 1991)
LMX1B	LIM homeobox transcription factor 1β	AD	Nail patella syndrome	(Dreyer, Zhou et al. 1998)
PAX2	Paired box gene 2	AD	Adult-onset FSGS, Renal coloboma syndrome	(Barua, Stellacci et al. 2014), (Amiel, Audollent et al. 2000)
MAFB	MAF BZIP Transcription Factor B	AD	Carpo-tarsal osteolysis progressive ESRD	(Zankl, Duncan et al. 2012)
LMNA	Lamin A/ C	XD	Familial partial lipodystrophy, FSGS	(Thong, Xu et al. 2013)
NXF5	Nuclear RNA export factor 5	XR	SRNS/FSGS cardiac conduction disorder	(Esposito, Lea et al. 2013)
NUP93	Nucleoporin 93kD	AR	Infantile SRNS	(Braun, Sadowski et al. 2016)
NUP205	Nucleoporin 205kD	AR	Infantile SRNS, BAV	(Braun, Sadowski et al. 2016)
XPO5	Exportin 5	AR	Infantile SRNS	(Braun, Sadowski et al. 2016)
NUP107	Nucleoporin 107kD	AR	Early childhood-onset SRNS/FSGS	(Miyake, Tsukaguchi et al. 2015)
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1	AD	Schimke immuno-osseous dysplasia	(Boerkoel, Takashima et al. 2002)

Encoding GBM proteins and related proteins				
LAMB2	Laminin subunit β2	AR	CoNS (either isolated or as an aspect of Pierson syndrome)	(Zenker, Aigner et al. 2004)
ITGB4	Integrin β4	AR	Epidermolysis bullosa	(Vidal, Aberdam et al. 1995)
ITGA3	Integrin α3	AR	Epidermolysis bullosa, Interstitial lung disease, SRNS/FSGS	(Nicolaou, Margadant et al. 2012)
CD151	CD151 tetraspanin	AR	Epidermolysis bullosa, Sensorineural deafness, ESRD	(Karamatic Crew, Burton et al. 2004)
Encoding mitochondrial proteins				
COQ2	4-hydroxybenzoate-polyprenyltransferase, mitochondrial	AR	Early onset SRNS, coenzyme Q_{10} deficiency	(Diomedi-Camassei, Di Giandomenico et al. 2007)
COQ6	Ubiquinone biosynthesis monooxygenase COQ6	AR	SRNS with sensorineural deafness	(Heeringa, Chernin et al. 2011)
PDSS2	Decaprenyl-diphosphatesynthase subunit 2	AR	SRNS, coenzyme Q_{10} deficiency	(Lopez, Schuelke et al. 2006)
ADCK4	AarF domain containing kinase 4	AR	Childhood-onset SRNS, CoQ10 deficiency	(Ashraf, Gee et al. 2013)
MTTL1	Mitochondrial Leu (UUR) tRNA	AR	MELAS, diabetes, deafness and glomerular lesions	(Goto, Nonaka et al. 1990)
Encoding lysosomal proteins				
SCARB2	Lysosome membrane protein 2	AR	Action myoclonus and renal failure	(Berkovic, Dibbens et al. 2008)
Encoding other proteins				
PLCE1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase ε1	AR	Infantile and early childhood SRNS	(Hinkes, Wiggins et al. 2006)
PTPRO	Receptor-type tyrosine-protein phosphatase O	AR	SRNS	(Ozaltin, Ibsirlioglu et al. 2011)
EMP2	Epithelial membrane protein 2	AD	Childhood SRNS/SSNS	(Gee, Ashraf et al. 2014)
DGKE	Diacylglycerol kinase-ε	AR	Atypical hemolytic uremic syndrome, membranoproliferative lesions	(Lemaire, Fremeaux-Bacchi et al. 2013)

Abbreviations: CoNS, congenital nephrotic syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes.

Genetic susceptibility factors and oligogenism

Several genetic susceptibility factors for FSGS have been identified in recent years through genome-wide association studies (GWAS) or other high throughput techniques. GWAS permits analysis of the genome in large populations of interest and may provide evidence for non-rare variants linked to specific traits. The most significant advance in this field was the discovery of the *apolipoprotein L1* (*APOL1*) risk variants for FSGS in African Americans with HIV or hypertension (Genovese, Friedman et al. 2010). The risk alleles, commonly designated *G1* and *G2*, are enriched in West Africa originating populations as a result of the resistance to Trypanosomiasis in carriers of one of the alleles (Thomson, Genovese et al. 2014). However, the presence of both alleles greatly increases the risk of primary FSGS (odd ratio (OR) =17), HIV associated FSGS (OR=29) and hypertensive nephropathy (OR=7) in African Americans (Genovese, Friedman et al. 2010, Kopp, Nelson et al. 2011). The presence of two risk alleles is frequent in this population (13%) and can account for the high prevalence of FSGS and chronic kidney disease which has long been known to be characteristic of this ethnic group. The mechanisms behind the increased risk are still not completely understood but recent studies suggest that it could be related to activated inflammasomes, endoplasmic reticulum (ER) stress or autophagy defects (Heymann, Winkler et al. 2017).

In another study, researchers investigated the genetic susceptibility to FSGS on a population of European ancestry, where APOL1 variants are quite rare (0.03%). They used a combined approach of gene panel high-throughput sequencing, targeting 2.500 podocyte expressed genes, and functional testing in mice with a susceptible genetic background, that could model FSGS as an oligogenic disease (Yu, Artomov et al. 2016). These mice were heterozygous for two podocyte genes, *Cd2ap* and *Synpo*, and developed FSGS in 25-50% of cases at 6 months (Huber, Kwoh et al. 2006). Six potential FSGS susceptibility genes (*WNK4*, *DLG5*, *KAT2B, KANK1*, *KANK2*, and *ARHGEF17*) identified by gene panel sequencing were silenced using a podocyte-specific and doxicycline inducible transactivator of the specific shRNA in the double heterozygous *Cd2ap*, *Synpo* mice. The development of significantly higher proteinuria with the silencing of the candidate gene allowed the identification of *WNK4*, *KANK1*, and *ARHGEF17* as new FSGS susceptibility genes (Yu, Artomov et al. 2016). *KAT2B* silencing led to a trend for higher proteinuria but not reaching statistical significance, as well as areas of podocyte foot process effacement. Interestingly *KANK1* and *KANK2* recessive mutations had previously been identified in several families with steroid-sensitive or steroid-dependent NS (Gee, Zhang et al. 2015).

These studies highlight the importance of considering oligogenic inheritance and genetic epistasis to, at least partially, explain intrafamilial phenotypic variability. In recent years these cases have come

into focus due to the increased availability of next generation sequencing (NGS) techniques which have allowed sequencing of multiple genes. For example, in a family diagnosed with Alport syndrome due to hemizygous mutations in *COL4A5*, the early onset and severity of the disease in two cases in the same family led to the discovery of additional homozygous mutations in *MYO1E* a known podocytopathy gene (Lennon, Stuart et al. 2015). Moreover, in several cohorts of SRNS patients, heterozygous variants in one additional SRNS gene were also found for a few affected individuals (Lowik, Levtchenko et al. 2008, Voskarides, Arsali et al. 2012, Bullich, Trujillano et al. 2015) and in some cases associated with a more severe phenotype, suggesting the existence of genetic epistasis (Voskarides, Arsali et al. 2012, Bullich, Trujillano et al. 2015). Outside the kidney field there are several examples of digenic or oligogenic inheritance. Analysis of 11 known genes of congenital hypothyroidism by NGS revealed oligogenic involvement in 39 of 177 unrelated patients, but no enrichment on rare variants in these genes were found in 3.583 controls (de Filippis, Gelmini et al. 2017). Margolin et al. reported that the syndrome of hypogonadotropic hypogonadism, ataxia, and dementia can be caused by the combination of mutations in *RNF216* and *OTUD4*, both encoding proteins related to ubiquitination, and leading to a more severe disease than *RNF216* mutations alone (Margolin, Kousi et al. 2013). Others have reported digenic inheritance on facioscapulohumeral muscular dystrophy type 2 (Lemmers, Tawil et al. 2012). In this case, a mutation in *SMCHD1,* encoding the protein structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1), leads to an epigenetic modification on the previously known disease locus *D4Z4*-array and to increased expression of the encoded protein DUX4, resulting in facioscapulohumeral dystrophy (Lemmers, Tawil et al. 2012). Recently, another group described digenic inheritance for hypoplastic left heart syndrome. Interestingly, in this case, potentially causal genes were evidenced by a genetic screen in mice, and two of these genes were then found to be mutated in one affected patient (Liu, Yagi et al. 2017).

Interestingly, oligogenism may not only be the cause of a more severe disease phenotype of a well defined clinical entity, but may also explain the existence of other phenotypic manifestations outside the known spectrum for a given disease. In fact, recently, Posey et al. reviewed 2076 patients referred for whole-exome-sequencing and found that 4.9% of the patients had two to four molecular diagnostics, involving known disease associated genes, each leading to a different phenotype (Posey, Harel et al. 2017). Others have also reported rare “syndromes” where the phenotype is actually the result of mutations in two different and unrelated genes. This is the case of the very rare “Fitzsimmons syndrome” (Armour, Smith et al. 2016) and may also apply to other syndromes reported in OMIM database, which do not have a molecular diagnosis. Due to the existence of

oligogenism, some authors state that reports of “phenotype expansion” of a rare disease can be sometimes the result of co-occurrences of mutations in different genes (Boycott and Innes 2017).

While genes already associated to well defined clinical entities may be easily identified by exome sequencing and the resulting phenotype clearly delineated in patients, even in cases of oligogenism, the finding of variants in genes previously not linked to a disease poses a big challenge. For this, using large-scale international data sharing may prove to be of great value, allowing the identification of very rare unknown variants and their common phenotypes. This is the case of Genematcher (https://genematcher.org/) (Sobreira, Schiettecatte et al. 2015) or Matchmaker Exchange 7 (http://www.matchmakerexchange.org/) (Philippakis, Azzariti et al. 2015).

Mendelian forms: Non-syndromic *vs* syndromic SRNS

In monogenic SRNS, mutations in specific genes, such as *NPHS1*, *NPHS2*, *PLCE1*, *ACTN4*, *INF2*, *TRPC6*, *MYO1E* or *ADCK4*, among others, result in a kidney restricted phenotype, and are classified as non-syndromic SRNS. However, mutations in one single gene can also lead to additional organ involvement besides the kidney. When multiple organs are affected we can speak of syndromic SRNS. Extra-renal manifestations may be even more prominent or present earlier than the kidney defect itself. In most cases the affected organ is the central nervous system, but the genital tract, eye, muscle, heart, bone and the immune system can also be affected.

There are multiple genes associated with syndromic SRNS, detailed in **Table 1**, including genes encoding transcription factors such as *WT1* and *LMX1B*, chromatin remodeling proteins such as *SMARCAL1*, proteins from the nuclear envelope *NXF5* or *NUP*93, mitochondrial proteins, i.e. *COQ2*, *COQ10*, *PDSS2*, or the mitochondrial leucine tRNA encoded by *MT-TL1*, lysosomal proteins such as *SCARB2*, GBM components or adhesion molecules, such as *LAMB2* and *ITGA3*, and some cytoskeleton proteins such as *WDR73*, *INF2* and *MYH9*. Multiple cellular pathways are thus associated to podocytopathies, underlining the complexity of these cells and the importance of considering other biological pathways besides the ones directly linked to the actin cytoskeleton or the slit diaphragm.

In some instances the affected organs correspond to the expression pattern of the mutated gene, as occurs for LAMB2. However, this is not always the case. For example, mutations in genes that are ubiquitously expressed may result in only a podocyte specific effect, as for *ACTN4*, *TRPC6*, *MYO1E* or *ADCK4* genes (Kaplan, Kim et al. 2000, Winn, Conlon et al. 2005, Sanna-Cherchi, Burgess et al. 2011, Ashraf, Gee et al. 2013), or affect just a few other organs besides the kidney, such as *INF2* (neuropathy) (Brown, Schlondorff et al. 2010), *NFX5* (cardiac conduction defects) (Esposito, Lea et al. 2013) or *WDR73* (neurological defects) (Colin, Huynh Cong et al. 2014). This finding may be due to the absence of redundant or modifier pathways in the affected cell or tissue, or alternatively the affected cell/tissues may have a particularly high dependence on the implicated function or pathway.

NGS has allowed to better understand the complexity of the syndromic forms of SRNS and also highlighted its phenotypic variability. This is the case for the mutations found in the transcription factor WT1. Although classically associated to Frasier and Denys Drash syndromes, with prominent involvement of the kidney in both sexes and genital tract particularly in males (Pelletier, Bruening et al. 1991, Barbaux, Niaudet et al. 1997), recent cases of isolated SRNS have been identified, where no gonadal involvement is reported in XY patients (Mucha, Ozaltin et al. 2006).

NGS and the need for high-throughput functional testing in model organisms

Classically, studies aiming to identify new candidate genes associated with rare diseases have used linkage analysis to distinguish the disease locus, followed by Sanger sequencing of the genes within the locus in order to determine the gene harboring the disease causing mutations. The success of this type of research was based on the presence of a sufficient number of genetically informative kindred that could narrow the genetic locus to a manageable length for direct Sanger sequencing. Rare and potentially pathogenic variants that were found using this strategy were usually limited to one gene. Moreover, due to the high number of affected individuals within one or several families that had allowed the establishment of a small disease locus, there was such a strong genetic basis that not much doubt remained regarding their causality. Examples of the strength of this strategy are the discovery of *NPHS1* (Kestila, Lenkkeri et al. 1998) and *NPHS2* (Boute, Gribouval et al. 2000) genes, or *INF2* (Brown, Schlondorff et al. 2010) and *TRPC6* (Winn, Conlon et al. 2005), responsible for most cases of AR or AD SRNS, respectively.

Compared to the classic technique of Sanger sequencing, massive parallel NGS methods now offer faster sequencing of a targeted group of genes (gene panel sequencing), the coding part of the genome by whole exome sequencing (WES), or the entire genome by whole genome sequencing (WGS). In clinical practice diagnostic panels of known genes associated to SRNS are routinely used and offer similar accuracy to Sanger sequencing with vastly superior efficiency and speed and inferior cost.

WES or WGS are the techniques of choice for cases where no mutation has been found in known SRNS genes, hence justifying the search of potential pathogenic variations in new candidate genes. These techniques allow the fast and cost effective sequencing of thousands of genes in families that were previously insufficiently informative from a genetic point of view. Since 2009 (Ng, Turner et al. 2009), this great technological advance has allowed the discovery of mutations in multiple novel genes for Mendelian diseases, and specifically in SRNS.

However, variants found using these methods in single families with a small number of affected individuals with a presumably hereditary disease must be interpreted with caution. In fact, the human genome has a huge number of rare and private variants (Coventry, Bull-Otterson et al. 2010, Lupski, Belmont et al. 2011) and determining their pathogenicity is challenging. Although there are several statistical tools and online databases and softwares that help analysing and prioritizing variants, such as SIFT (http://sift.jcvi.org/), Polyphen (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) and SDM

(http://mordred.bioc.cam.ac.uk/~sdm/sdm.php) (Liu, Jian et al. 2011, Petrovski, Wang et al. 2013, Lek, Karczewski et al. 2016), understanding the pathogenicity of the variants relies on functional studies. This highlights the need for the development of model organisms that ideally allow a fast high-throughput analysis of the variant pathogenicity.

Mouse models have been used extensively in renal research and are clearly a powerful model organism. However, murine studies are quite time consuming and expensive, even with the new regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) technique. Moreover the analysis of more complex genetic traits can prove to be even more difficult in this model. In addition, although mice and humans have a high genetic homology, there are several examples where no phenotype has been found in mice despite the fact that mutations in the gene are actually disease causative in humans, namely for *PLCE1* (Wang, Oestreich et al. 2005). This may be due to different tissue expression pattern in rodents or to redundant functions between paralog genes that can be completely overlapping in mice but less in humans.

The Zebrafish is an alternative vertebrate model organism. In fact, renal research in the recent decades has highlighted strong similarities with the mammalian renal system as well as its potential to study kidney genetic diseases and renal regeneration. Its main disadvantage may be their genome duplication that can lead to a high degree of redundancy between genes, complicating KO studies.

In the next chapter I will expose the advantages of the *Drosophila* model for the study of genetic renal diseases, in particular podocytopathies.

The *Drosophila* model as a tool to study podocytopathies and more…

Drosophila for the study of human diseases

D. melanogaster is a powerful model organism. Its short life cycle (10 days at 25ºC) (**Figure 12**) allows for the rapid generation of transgenic flies, to efficiently perform genetic combinations, and to generate a large number of individuals. The cost of keeping fly stocks is much lower than that of other model organisms such as mice or zebrafish. Moreover, since the beginning of the 20th century, a large array of molecular biological tools has been developed for this model organism.

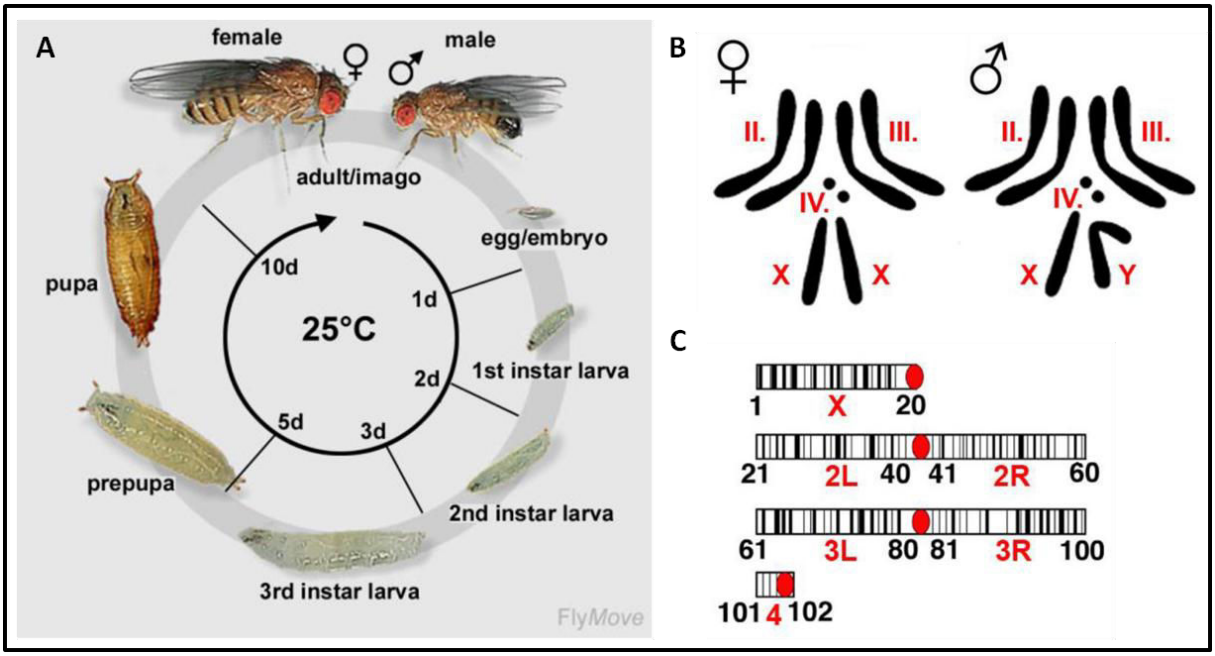

Figure 12. The life cycle of *Drosophila melanogaster* and its chromosomes. (A) At 25°C the life cycle of fruit flies is around 10 days: Embryonic development lasts 21h; hatched larvae (1st instar) take 1 day to molt into 2nd instar larvae and these take 1 more day to molt into 3rd instar larvae; 3rd instar larvae pupariate at the 5th day of the life cycle and undergo metamorphosis; 10 days after, adult flies emerge from the pupal case and are sexually mature after 8 hours. These times are doubled when flies are raised at 18°C. **(B)** Representation of *Drosophila* male and female mitotic chromosomes, showing one pair of heterosomes and three autosomal chromosomes (II-IV). **(C)** Schematic representation of *Drosophila* polytene chromosomes, which have a banding pattern that is used for cytogenetic mapping of gene loci (black numbers). The 2nd and 3rd chromosomes are further divided in left (L) and right (R) by the centrosome (dot). Adapted from Prokop *et al.* 2013.

In *Drosophila* a high degree of phylogenetic conservation is known for genes that control fundamental biological processes. This was extended to human disease genes by a study where a detailed cross-genomic analysis was done between all human disease genes in OMIM (Online Mendelian Inheritance in Man, https://www.omim.org/) and the *Drosophila* genome. The authors found that 75% of human disease genes have a homolog in flies (Reiter, Potocki et al. 2001).

Compared to humans, flies have a lower genomic redundancy, i.e. only one or few genes code for several members of a protein family in mammals. Interestingly, it has been shown that genes that are essential for viability in *Drosophila* and have several human homologs are much more likely to cause Mendelian diseases (Yamamoto, Jaiswal et al. 2014). These authors propose that when a single essential fly gene has multiple copies in humans, the homologs will have partially redundant functions and are therefore not likely to be embryonic lethal in humans but prone to a particular disease upon loss of function (**Figure 13**). Nevertheless, this rule does not apply in all cases, as in several late onset degenerative diseases, such as Parkinson or Alzheimer, the homolog fly gene is not essential for fly viability.

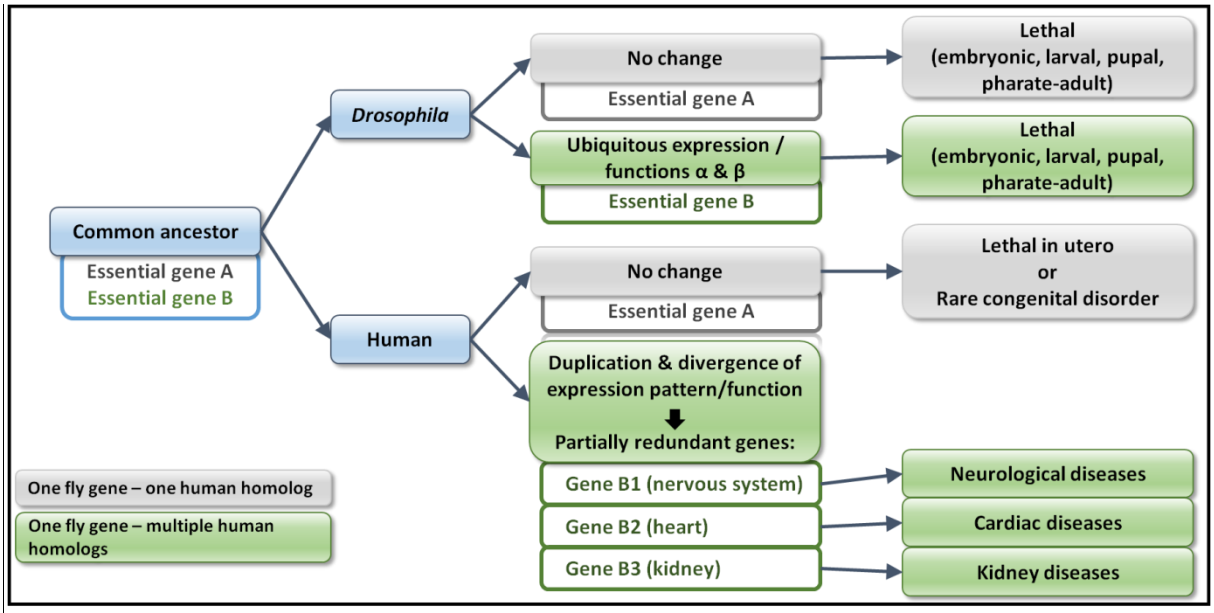

Figure 13. Simplified evolutionary patterns of essential genes and their association with Mendelian disorders. Evolutionary relationships between essential fly genes and human genes based on common ancestry. In the cases of genes with only one human homolog (grey, gene A), these are likely to play essential roles in flies and in humans leading to early lethality or very severe congenital diseases. For conserved essential genes in which one fly gene is related to multiple homologs in humans (green genes, B1,B2,B3) there is enrichment of organ specific diseases due to tissue specific expression in vertebrates and/or because each vertebrate homolog takes non-redundant functions. Adapted from Wangler *et al.* 2015.

In general terms, there are three main ways of studying human disease in flies: 1) reverse genetics, 2) diagnostic strategy (recently established to help discover human disease-causing genes) and 3) forward genetics (Ugur, Chen et al. 2016) (**Figure 14**).

Reverse genetics is the approach used to discover the functions of genes of interest and study their phenotypes *in vivo*. It applies to cases where the desired outcome is the understanding of the molecular mechanisms or functions of genes that are associated with human diseases. For this purpose loss or gain of function studies are used, either creating new mutants or using already existing mutants and transgenic flies available at international stock centers (i.e. Bloomington

Drosophila Stock Center (BDSC), Vienna *Drosophila* Resource Center (VDRC)). In addition, the pathogenic mechanism of specific human mutations can be assessed by overexpression of the WT or the mutant gene in a specific tissue (Feany and Bender 2000) or more recently using the CRISPR/Cas9 strategy.

Diagnostic genetics correspond to a subtype of reverse genetics. In this case the *Drosophila* model is used to assess the pathogenicity of new mutations that have been identified in human diseases (either in new candidate genes or in already known candidate genes, if the pathogenicity of the mutations is not established). First, the expression of the fly homolog is silenced and the phenotype is assessed. Subsequently the WT or the human or fly gene harboring the identified mutation is expressed and causality is determined if the rescue is achieved by the WT but not the mutated gene (Bellen and Yamamoto 2015, Wangler, Yamamoto et al. 2015).

Forward genetics on the other hand, begins with a large unbiased screen in flies using RNA interference (RNAi) screens, chemical mutagenesis (ethylmethanesulfonate (EMS)) or transposon-mediated mutagenesis, and then looking for a specific phenotype of interest. Subsequently mutated genes that lead to the phenotype of interest are identified using libraries of genetic deletions that cover the whole fly genome. This strategy can help to identify new genes linked to a disease or discover unsuspected underlying biological mechanisms.

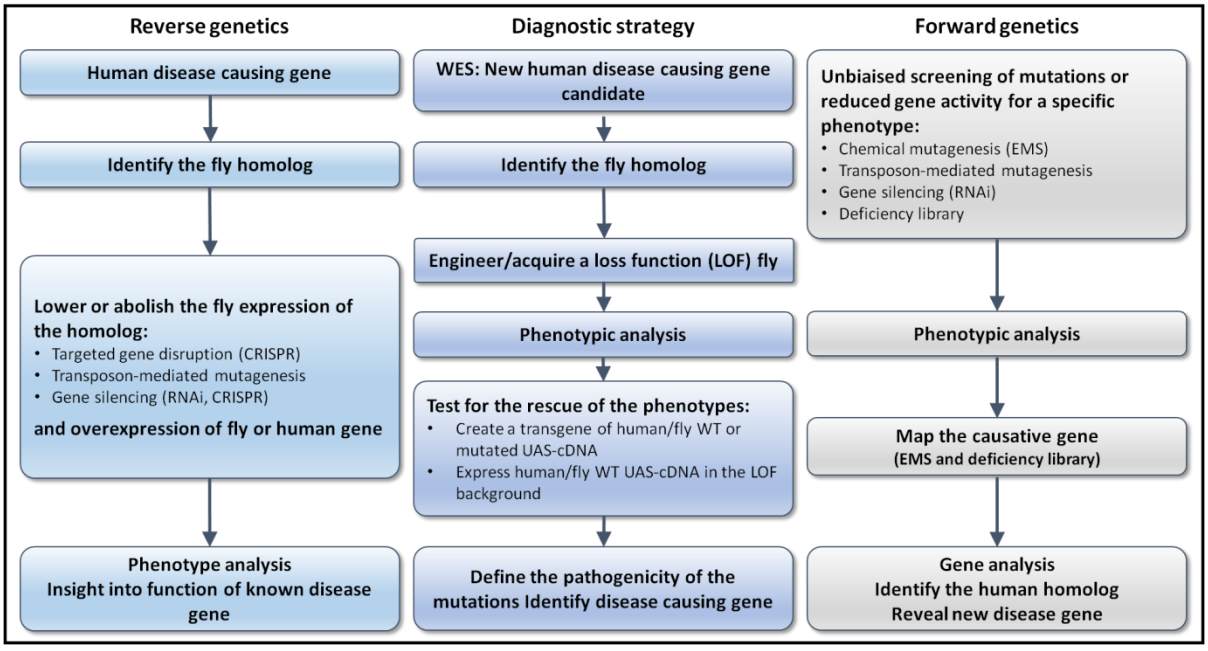

Figure 14. Genetic strategies used in *Drosophila* for the study of human diseases. Three types of strategies are proposed and described in the schema. New human disease candidate genes and mutations found by whole exome sequencing (WES) can be studied using the diagnostic strategy. Adapted from Ugur *et al.* 2016.

Besides assessing the pathogenicity of a mutation or understanding the molecular mechanisms of a disease, the fly model can also be used to perform second site modifier screens in order to identify loci that may enhance or suppress the disease phenotype (Pagliarini and Xu 2003). This may be particularly helpful for studying diseases with variable expressivity or penetrance, where oligogenism is suspected. Moreover, flies can be used for drug screening of large libraries of chemical compounds (Steffan, Bodai et al. 2001).

In flies, observation of cells and tissues *in vivo* or *ex vivo* is relatively easy due to the small organ size and low complexity. Analysis of cardiac disease in flies, for instance, can be feasible. The simple anatomy of the fly heart, a heart tube, although not suitable to model problems specific to the 4 chambers of mammalian hearts, has a very conserved mechanism of development through evolution and is experimentally amenable to physiological testing and manipulation (Wessells and Bodmer 2004, Wessells, Fitzgerald et al. 2004).

All these factors have allowed the fly model to be successfully used for the discovery of mechanisms underlying several human diseases, such as developmental disorders, neurological disorders and cancer (Bier 2005).

Genetic Tools in *Drosophila*

Of all animal models, *Drosophila* has one of the most advanced molecular genetics tool kits. The fly genome was one of the first to be sequenced and all genes are annotated in public databases (FlyBase; http://flybase.org/). Tools include transgenesis with transposable elements, targeted gene expression systems such as the bipartite GAL4:UAS, libraries of genetic deletions covering the whole fly genome, RNAi constructs (Mohr 2014) for virtually all *Drosophila* genes, as well as the recent CRISPR/Cas9 that can allow the targeted disruption of a gene and the knock-in of mutations (Beumer and Carroll 2014) (**Figure 15**). Transgenic flies carrying a genetic construct of interest can also be obtained by using the PhiC31 integrase system. A plasmid containing the gene or cDNA of interest flanked by attB sites and a phenotypic marker is injected into an attP-containing docking site fly strain with PhiC31 activity. Phi-C31 integrase then mediates sequence directed irreversible integration of the genetic construct and mutants are selected through the presence of the phenotypic marker.

Cell type-specific gene manipulation through the use of the GAL4:UAS system is also possible (Brand and Perrimon 1993). GAL4:UAS is a bipartite expression system that relies on transcriptional activation by the yeast protein GAL4 to express a genetic construct placed downstream of multiple

GAL4 binding sites (**Figure 15**). Large collections of different promoter elements driving the expression of GAL4 (drivers) are available and allow the expression of the genetic construct in a tissue- or even cell-specific pattern. Through manipulation with molecular switches (i.e. GAL80 suppressor) gene expression can also be turned on or off. This allows a temporally controlled switch of the gene of interest and hypotheses can be tested even in the context of a lethal phenotype.

Another advantage of *Drosophila* is the existence of balancer chromosomes, these are genetically modified chromosomes, lethal or causing sterility in homozygosis, that do not allow recombination and that carry easily identifiable phenotypic markers. Balancer chromosomes thus allow to maintain a stock with mutations that would be otherwise lethal, or to know the genotype of flies from a mating scheme by looking to the phenotypic markers, without having to perform classic genotyping.

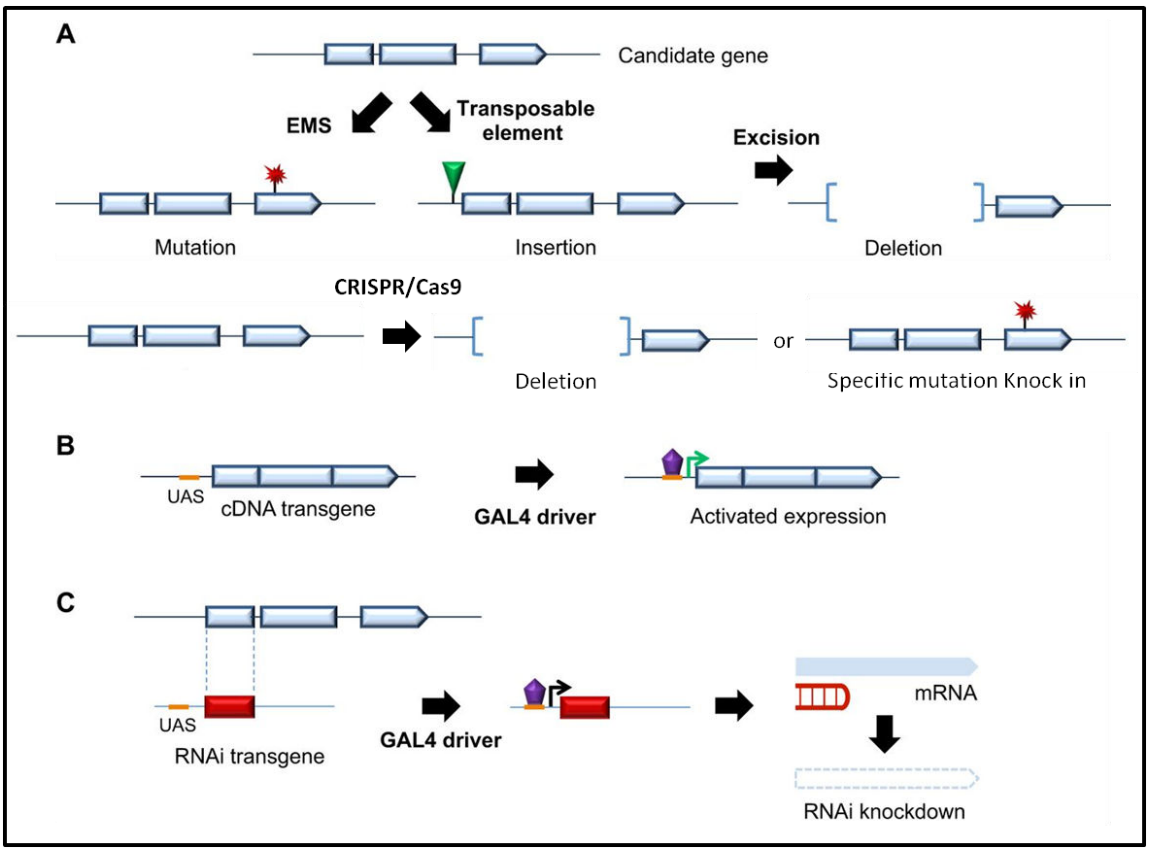

Figure 15. Some of the strategies for genetic manipulation in *Drosophila*. (A) Loss of function (LOF) alleles for a candidate gene can be obtained from large public *Drosophila* collections and from other laboratories. Chemical mutagenesis with EMS leads to point mutations (red flash). Transposable elements insertions (green triangle) can lead to gene disruption or deletion alleles via imprecise excision. More recently CRISPR/Cas9 strategy has also been used to create LOF alleles or to introduce specific mutations in the genes of interest, allowing to study the effect of these variants at endogenous levels of protein expression. **(B)** GAL4:UAS binary system for expressing a gene of interest with spatial and temporal control. Transgenic flies carry a cDNA for the gene of interest, under the control of the yeast upstream activating sequence (UAS). A huge collection of 'driver lines' are available, which express the yeast GAL4 transcriptional activator (purple) in different tissues. A genetic cross brings these elements together, activating the expression of the cDNA when the GAL4 binds to the UAS sequence. **(C)** GAL4:UAS binary system for the KD of a gene via RNA interference (RNAi). Transgenic RNAis strains for nearly all *Drosophila* genes are available in public stock collections. The GAL4 driver will define the tissue specificity of the KD. Adapted from Wangler *et al.* 2017.

The *Drosophila* excretory system – an analogy to the human kidney

The renal excretory system in flies is considered to be composed of nephrocyte cells that filter hemolymph and then endocytose and metabolize the filtered macromolecules, and the Malpighian tubules, which excrete ions and water, regulating the fly hydro-electrolytic equilibrium (Helmstadter and Simons 2017) (**Figure 16**). Contrary to the vertebrate kidney where these two functions are coupled in one sole unit, the nephron, these two components are physically separated in flies. Functionally, the nephrocyte represents the podocyte and the first segment of the mammalian tubule (proximal tubule), responsible, respectively, for filtration and the reabsorption of small molecular weight proteins and other small molecules that pass the GFB. The Malpighian tubule on the other hand, represents the more distal parts of the nephron that finely regulate the water and ion balance by complex ion pump dependent mechanisms. This thesis will mainly focus on the nephrocyte and its similarities to the podocyte.

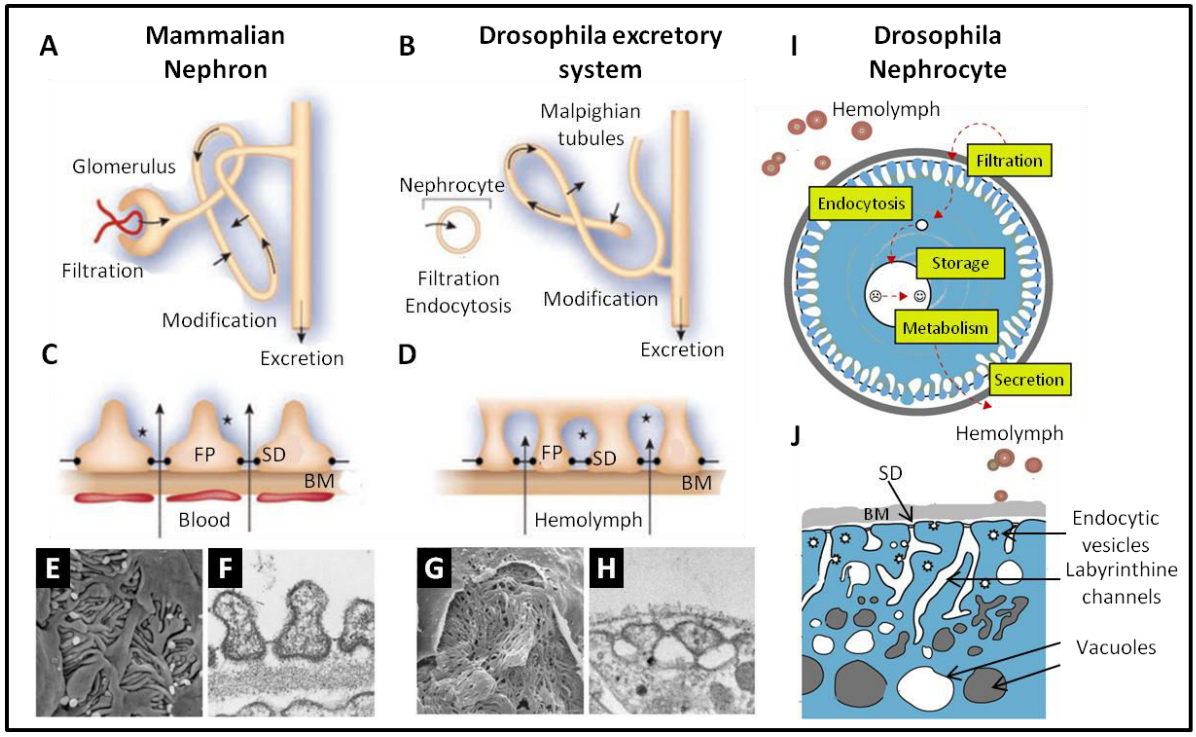

Figure 16. Homology between human and *Drosophila* excretory systems. (A,B) Schematic drawings of the mammalian nephron and the *Drosophila* excretory system. In *Drosophila,* filtration and endocytosis occurs in the nephrocyte while fluid and ion secretion is performed by the Malpighian tubule. **(C,D)** Schematic drawings of foot processes (FP) and slit diaphragms (SD) of podocytes (left) and nephrocytes (right), highlighting their similarity. **(E-H)** SEM and TEM pictures of the mammalian nephrocyte (mouse samples) **(E,F)** and the *Drosophila* nephrocyte (**G,H**). (**I**) Main functions of the nephrocyte include filtration of hemolymph followed by endocytosis of low molecular weight proteins and toxins that undergo further metabolism or storage in the nephrocyte. (**J)** Detailed schematic of the nephrocyte with its basement membrane (BM), SD and deep labyrinthine channels. Note the active endocytic machinery at the foot process lateral membranes leading to the formation of prominent "vacuoles". Adapted from Simons *et al.* 2009 and Denholm *et al.* 2009.

The nephrocyte and the nephrocyte filtration barrier

Although nephrocytes have been known since the end of the 19th century (Kovalevskij 1892), it has been less than a decade since these cells were proposed to be podocyte-like cells with a slit diaphragm. It was in 2009 that Weavers et al., followed by Zhang et al. in the same year, performed a detailed ultrastructural, molecular and functional study of the nephrocyte (Weavers, Prieto-Sanchez et al. 2009, Zhuang, Shao et al. 2009). Nephrocytes were found to have extensive invaginations of the plasma membrane that create the so-called "labyrinthine channels" and, between them, structures analogous to the podocyte foot processes (**Figure 16**). These structures are linked by slit diaphragms that are composed of the homologous proteins of the mammalian slit diaphragm. These comprise the imunoglobulin-superfamily proteins Kin-of-irre (Kirre) corresponding to Neph1 and Sns (Sticks-and-stones) corresponding to nephrin, as well as slit diaphragm adaptor proteins such as Mec2, corresponding to podocin, Cindr corresponding to CD2AP and other partners described in **Figure 17**.

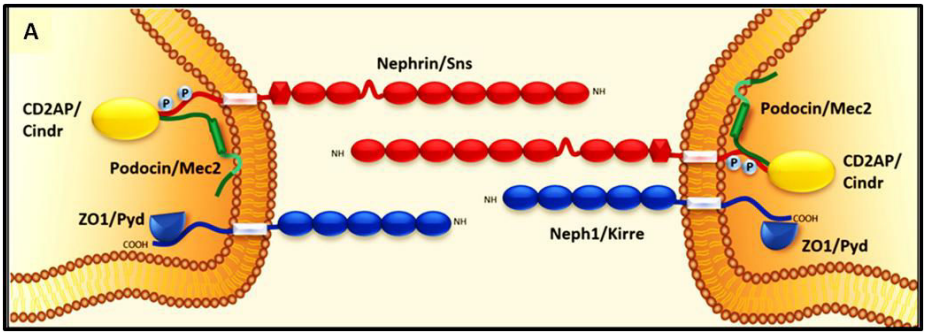

Figure 17. Molecular homology between the components of the podocyte and the nephrocyte slit diaphragm. Shown are the main components of both the human and *Drosophila* slit diaphragm. The correspondence between the human and the fly homologs is depicted in the figure. Adapted from Helmstädter *et al.* 2017.

The interactions of these proteins in *Drosophila* closely replicate the mammalian ones. Interestingly Sns, Kirre and related proteins from the imunoglobulin-superfamily serve as cell-recognition molecules and participate in several cellular interactions, such as myoblast fusion, axonal pathfinding and target recognition, controlling the exact positioning of primary pigment cells and interommatidial cells in the compound eye. Loss of one of these homologs leads to misalignment of the ommatidia and a "rough" appearance of the eye (Fischbach, Linneweber et al. 2009).

The nephrocyte slit diaphragm has a size selective permeability, discriminating between molecules of different size and allowing the passage of small molecular proteins, up to a recently defined cut-off of around 70KDa (Zhang, Zhao et al. 2013, Hermle, Braun et al. 2017), as in mammals. The nephrocyte slit diaphragm is of approximately the same size of the mammalian one, around 30nm (Weavers, Prieto-Sanchez et al. 2009). Besides the slit diaphragm, it has been shown that the basement membrane that surrounds these cells is negatively charged, also participates in filter

functions and is composed of collagen type IV produced by the nephrocyte (Hermle, Braun et al. 2017), as is the case in the mammalian GFB.

The nephrocyte endocytic machinery

After filtration by the slit diaphragm, macromolecules are further endocytosed in the nephrocyte "labyrinthine channels" or "lacunae". These have a tubular structure with multiple subdivisions that create a large membrane surface, known to have a highly active endocytic machinery, first described in 1972 (Wigglesworth 1972). Endocytosis *is* dynamin dependent for soluble macromolecules that pass the nephrocyte filter and seems to be dependent on a process of macropinocytosis regulated by Rudhira, a nephrocyte specific cytoplasmic WD40 domain protein, for the uptake of colloidal material that could eventually occur at the tip of the foot processes (Kosaka and Ikeda 1983, Das, Aradhya et al. 2008). Following the labyrinthine channels, numerous clathrin coated vesicles, endosomes, vacuoles and lysosomes, organize in a circular fashion from the periphery to the center of the cell. Rab5- positive early endosomes can be observed on the very cell periphery and Rab7-positive later endosomes on a more central layer, followed by the lysosomes (Bechtel, Helmstadter et al. 2013, Lorincz, Lakatos et al. 2016) (**Figure 18**). RabGTPase function has recently been analyzed in a high throughput screen in *Drosophila* nephrocytes. About one third of *Drosophila* Rabs was shown to be important for normal nephrocyte activity. Rab5 silencing completely abolished all the intracellular vesicles and the labyrinthine channels. Gene silencing of Rab7 increased clear vacuoles and decreased lysosomes, suggesting a blockage later in the degradation pathway, and silencing of Rab 11 decreased clear vacuoles and increased the number of lysosomes, blocking as expected the recycling pathway (Fu, Zhu et al. 2017).

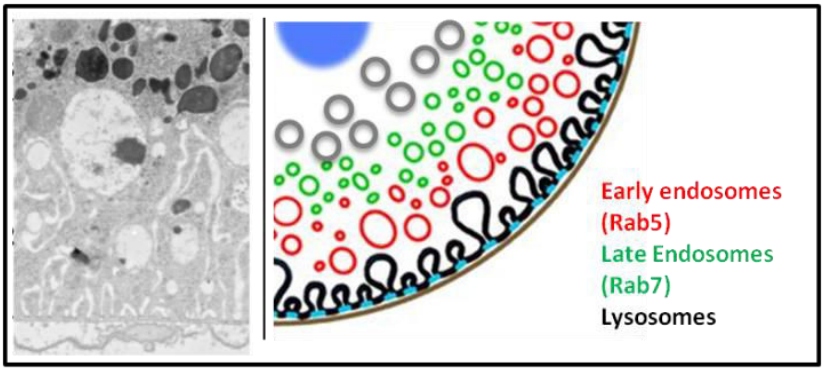

Figure 18. Distribution of endocytic vesicles in nephrocytes. TEM (left) and illustration (right) show the spatial organization of endocytic vesicles, that distribute from the cell periphery to the cell nucleus in early endosomes, late endosomes and lysosomes. Adapted from Fu *et al.* 2017 and Bechtel *et al.* 2013.

Interestingly, this highly active and dedicated endocytic machinery in nephrocytes has homologous receptors for protein reabsorption in the proximal tubule cells, cubilin and amnionless (Zhang, Zhao et al. 2013). This suggests that nephrocytes combine both podocyte and proximal tubule cell functions.

Nephrocyte subpopulations and development

Nephrocytes exist in two subpopulations: garland nephrocytes, which surround the esophagus, just above the proventriculus, and pericardial nephrocytes which flank the heart tube (**Figure 19**). In both cases they profit from the contracting movement of the adjacent organs, namely the esophagus and the heart, to have a dynamic contact with the hemolymph. This system can partially provide hemolymph flux and turnover necessary for an effective filtration, analogous to the pressure system in the capillary tuft of the glomerulus. Nephrocytes are physically fixed to the respective organs by their extracellular matrix and muscle fibers. For example, in the case of pericardial nephrocytes a dense extracellular matrix composed of laminin β, collagen IV α1 and the protein pericardin, a *Drosophila* α chain type IV collagen like protein (Chartier, Zaffran et al. 2002, Hollfelder, Frasch et al. 2014), is known to surround both the pericardial nephrocytes and the heart tube, and to be important for their correct alignment (Chartier, Zaffran et al. 2002, Hollfelder, Frasch et al. 2014). Moreover, alary muscles spread between and around the nephrocytes and heart, also having both support and alignment functions (LaBeau, Trujillo et al. 2009). Interestingly, in our TEM studies we could see that these muscle-like cells touch the nephrocytes, sharing their basement membranes at the points of contact.

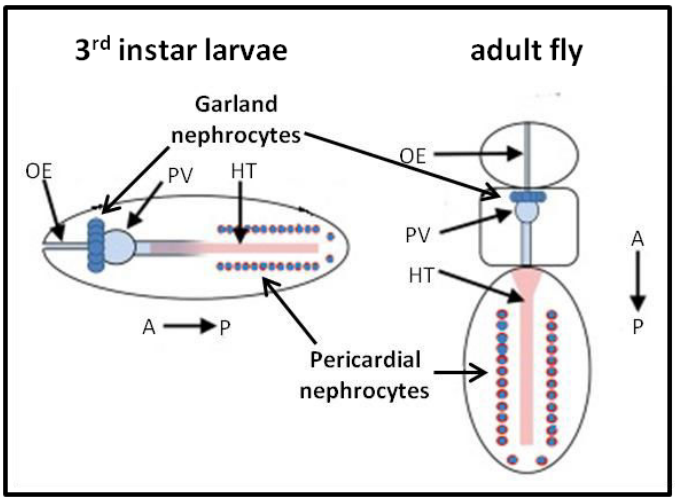

Figure 19. Nephrocyte subpopulations in *Drosophila*. Schematics showing the localization of garland nephrocytes and pericardial nephrocytes in larvae and adult flies. Garland nephrocytes can be found surrounding the oesophagus (OE), above the proventriculus (PV). Pericardial nephrocytes are on either side of the heart tube (HT). A (anterior) P (posterior). Adapted from Ivy *et al.* 2015.

Nephrocytes derive from the embryonic mesoderm, and although they decrease in number through the embryonic and larval stages, many persist into adult life (Sellin, Albrecht et al. 2006). For

example, there are approximately 30 pericardial nephrocytes at the adult stage (Sellin, Albrecht et al. 2006). Pericardial nephrocytes are derived from the cardiac mesoderm, that also gives rise to the heart and the lymph gland (the source of hemocytes, the *Drosophila* blood cells) (Mandal, Banerjee et al. 2004), and is dependent on signaling pathways that also govern differentiation in the mammalian aorta-gonadal-mesonephros mesoderm, such as Decapentaplegic (Dpp), fibroblast growth factor (FGF), Wingless (Wg) and Notch signaling (Mandal, Banerjee et al. 2004). The garland nephrocytes, that number about 25 in the larval stage (Aggarwal and King 1967) and are also present in adult flies, develop from the procephalic mesoderm cells which also give rise to hemocytes as well as a rudimentary cephalic aorta (de Velasco, Mandal et al. 2006). Interestingly, garland nephrocytes become binucleated at embryonic stage 16 due to a process of fusion that seems to be mediated at least in part by the Ig superfamily proteins, such as Kirre (Zhuang, Shao et al. 2009), already known to drive fusion mechanisms in myoblasts (Shelton, Kocherlakota et al. 2009). Interestingly the loss of Kirre or its over-expression leads to increased fusion in garland nephrocytes (Helmstadter, Luthy et al. 2012).

Several transcription factors have been implicated in nephrocyte viability, such as the basic Helix Loop Helix (bHLH) gene *Hand* (Han, Yi et al. 2006) and more recently the *Drosophila* homolog of the transcription factor KLF15, which is specific to both nephrocyte subpopulations. Loss of function of Klf15 (Ivy, Drechsler et al. 2015) leads to the loss of nephrocytes in larval and adult stages, even if they are initially present at the embryonic stage. Interestingly mammalian KLF15 has been shown to regulate renal fibrosis (Gao, Huang et al. 2011) as well as podocyte differentiation (Mallipattu, Liu et al. 2012). Moreover the polycomb gene complex is able to regulate the transcription factor Knot (corresponding to EBF2 in humans) that further regulates *Sns* (corresponding to *NPHS1* in humans) (Na, Sweetwyne et al. 2015).

The nephrocyte contribution to fly homeostasis and lifespan

One of the first functions ascribed to the nephrocytes was the capacity to store and/or secondarily metabolize toxic molecules *(Crossley 1985, Locke 1998).*

More recently, several authors have further demonstrated that dysfunctional nephrocytes due to genetically induced molecular defects were not able to store AgNO3 and that *Drosophila* larvae had reduced survival when exposed to medium containing this toxin (Das, Aradhya et al. 2008, Weavers, Prieto-Sanchez et al. 2009, Zhang, Zhao et al. 2013). However, no difference in larvae viability was seen with normal, non-toxic medium. Several studies have shown however a significant decrease in

the lifespan of adult flies with dysfunctional nephrocytes, even when not exposed to a particular toxin (Das, Aradhya et al. 2008, Fu, Zhu et al. 2017, Zhu, Fu et al. 2017). These results suggest that nephrocytes have a particularly important role in stress conditions and in ageing flies, but that animals can survive rather well without these cells. This is quite different to what happens in humans, where persistent podocytopathies lead to progressive glomerulosclerosis and loss of kidney function. Perhaps the physical separation of nephrocytes from Malpighian tubules permits the remaining cells to maintain fluid and electrolyte homeostasis, allowing fly survival in non-stress conditions.

Another interesting study showed that the absence of adult nephrocytes secondarily induced a cardiomyopathy (Hartley, Motamedchaboki et al. 2016). Analysis of the hemolymph proteome further showed a substantial impact on the quantitative and qualitative composition of hemolymph circulating proteins in flies with genetically induced loss of nephrocytes. Among these the matricellular protein Secreted protein acidic and rich in cysteine (Sparc) was one with the highest increase upon nephrocyte ablation. Interestingly Sparc is a protein involved in mammalian cardiac function, and the authors were able to show that reducing Sparc ameliorated the cardiomyopathy that developed in the absence of nephrocytes.

The nephrocyte as a model for human podocytopathies

Since the nephrocyte presents a high degree of homology with the podocyte, and as this cell plays a main role in all glomerulosclerosis processes, several efforts have been made to develop the fly nephrocyte as a new *in vivo* model for podocytopathies. Encouragingly, the KD of Kirre, homolog of Neph1, or Sns, homolog of Nephrin, frequently mutated in congenital NS, results in a decreased number of nephrocyte diaphragms and smoothening of the foot processes (Weavers, Prieto-Sanchez et al. 2009, Zhuang, Shao et al. 2009), resembling the foot process effacement that is seen in human podocytopathies (**Figure 20**). This also occurs with the KD of Pyd (Zhuang, Shao et al. 2009), the homolog of mammalian ZO-1, that is known to interact with Neph1 (Huber, Schmidts et al. 2003), with Mec2 (Zhang, Zhao et al. 2013), homolog of podocin, and with many other genes known to be mutated in genetic forms of the NS (Fu, Zhu et al. 2017, Hermle, Braun et al. 2017). The loss of slit diaphragms also leads to decreased length and number of labyrinthine channels and this finally results in a decrease of protein uptake (Weavers, Prieto-Sanchez et al. 2009, Zhuang, Shao et al. 2009, Zhang, Zhao et al. 2013, Hermle, Braun et al. 2017), probably due to a decrease in the available membrane surface for endocytosis.

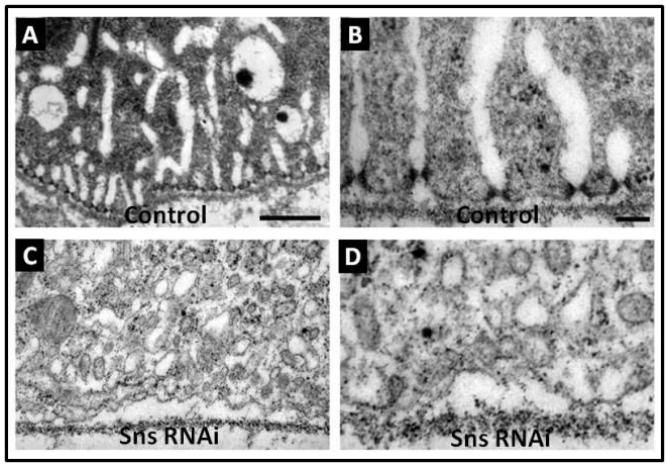

Figure 20. Nephrocyte foot process effacement. (A,B) In physiological conditions nephrocytes show well formed foot processes with visible slit diaphragms and deep labyrinthine channels **(C,D)** Upon Sns KD there is foot process effacement with simultaneous disruption of labyrinthine channels structure. Scale bars 500nm in A and C and 100nm in B and D. Adapted from Zhang *et al.* 2013.

It is interesting to note that the foot process effacement can also be achieved with the administration of puromycin aminonucleoside as demonstrated by Tutor et al. (Tutor, Prieto-Sanchez et al. 2014) and by protamine sulfate (Hermle, Braun et al. 2017), two commonly used drugs for inducing podocyte foot process effacement in mouse models. Moreover, the administration of a high sugar diet to flies is also able to induce nephrocyte defects, such as filtration defects and loss of Sns and Kirre, suggesting that these cells can also be used to study molecular aspects of diabetic nephropathy (Na, Sweetwyne et al. 2015).

Several read-outs have been used for screening the nephrocyte function, namely using endocytosis fluorescent tracers such as albumin and the uptake of toxins such as silver nitrate (Fu, Zhu et al. 2017, Hermle, Braun et al. 2017). More recently life-span experiments have also been used to assess the function of homologs of human genes mutated in SRNS (Fu, Zhu et al. 2017). Immuno-fluorescence can be used to assess nephrocyte differentiation markers, such as Kirre, Pyd, and Sns. Finally TEM can also be used to morphologically assess foot processes and slit diaphragms but is far more time consuming, being more useful to assess the effect of one or a small number of genes, but not for high-throughput screenings. In 2013 an elegant reporter system to evaluate nephrocyte filtration and endocytosis *in vivo* was developed by Zhang et al. (Zhang, Zhao et al. 2013). These flies express a fluorescent tagged secreted protein (ANF-RFP) that is produced in muscle and circulates in hemolymph being filtered and endocytosed by nephrocytes. The co-expression of GFP under the Hand promoter that is expressed in nephrocytes and cardiomyoblasts, allows easy identification of nephrocytes *in vivo* or *ex vivo* and quantification of the ANF-RFP uptake. Interestingly, several studies have also shown that the number of pericardial nephrocytes can decrease in response to structural abnormalities induced by *Cindr* KD, LOF alleles of the transcription factor *Klf15* or overexpression of the human *APOL1* risk alleles (Ivy, Drechsler et al. 2015, Na, Sweetwyne et al. 2015, Kruzel-Davila, Shemer et al. 2017), thus being an alternative read-out.

Two different studies performed a screen on known SRNS associated genes using the GAL4:UAS system to induce the conditional KD of the *Drosophila* homologs specifically in nephrocytes (Fu, Zhu et al. 2017, Hermle, Braun et al. 2017). The first one analyzed the homologs of 36 SRNS human genes, based on the ability of garland nephrocytes to filter FITC-albumin, and showed that 55% of these genes decreased albumin uptake at 3rd instar larval stage (Hermle, Braun et al. 2017). The second one studied the *Drosophila* homologs of 40 SRNS associated genes, and nephrocyte function was assessed using endocytosis of ANF-RFP, uptake of AgNO3 in young adult flies, fly survival and life span. These authors showed that nephrocyte defects were induced upon silencing of 83% of these genes (Fu, Zhu et al. 2017). The higher number of genes identified in the second screen could be due to the higher number and higher sensitivity of the methods used and to the fact that nephrocytes were studied at later time points, allowing an accumulating effect of a persistent lesion in nephrocytes, as could occur in late onset cases of SRNS. Common to both studies, the KD of the homologs of *PTPRO* and *PDSS2* did not give a phenotype in nephrocytes. This could be due to an insufficient silencing by the RNAi. Except for *ANLN* (encoding Anillin) that does not give a phenotype in the more extensive set of experiments performed on the second study while it shows a phenotype in the first study, all the other genes that had no phenotype in the first study were found to have at least one phenotype when silenced in the second study.

Drosophila nephrocytes have recently started to be used as functional models to test SRNS new candidate genes, as it was the case for the *KANK* genes (Gee, Zhang et al. 2015) and for *SGPL1* (ourselves (Lovric, Goncalves et al. 2017)) and mutation validation in known SRNS genes, such as *CD2AP* and *COQ2* (Fu, Zhu et al. 2017, Zhu, Fu et al. 2017). In these cases it is important not only to perform the assessment of phenotype in loss of function experiments but also to assess the impact of the patient mutations to prove their pathogenicity. This can be done by re-expressing the WT or the mutated form of the candidate gene on the KD / KO background using GAL4:UAS systems and specific nephrocyte or ubiquitous drivers. Alternatively, if the whole gene is cloned with its 5' upstream region, the endogenous promoter can directly drive the expression of the transgene. The CRISPR/Cas9 system may now easily allow the generation of a KO mutant (if one is not available from Flybase; http://flybase.org/) and to replace the fly gene by the human or fly WT or mutant rescue constructs, which in this case will be expressed under the endogenous promoter.

Whether to perform the rescue experiments using the human gene or its fly homolog depends on the degree of conservation, namely of the mutated amino acid residues. Although human disease-related genes globally have a good degree of conservation in flies, the phylogenetic distance between man and fly may in some instances preclude the rescue with the human gene or require the expression of the human multiple paralogs in the case of gene duplication.

Frequent disease risk alleles for glomerular diseases such as the G1 and G2 variants of the *APOL1* primate gene have also been studied in flies (Fu, Zhu et al. 2017, Kruzel-Davila, Shemer et al. 2017) and also proved the validity of this model for more common variants. In fact, overexpression of the *APOL1* risk variants in nephrocytes causes accumulation of ANF-RFP at early stages and nephrocyte loss at later stages in adult flies (Fu, Zhu et al. 2017, Kruzel-Davila, Shemer et al. 2017).

Finally, drug screening may also be possible in the nephrocyte model. In the rare variants of hereditary SRNS that have an identified therapy, such as those caused by mutations in the genes involved in the biosynthesis of COQ10, the administration of COQ10 to the fly food led to a partial rescue of the defects caused by the gene silencing in nephrocytes (Zhu, Fu et al. 2017).

Sphingolipids and kidney disease

Sphingolipids were first described in 1884 and were named after their enigmatic properties ("Sphinx-like")(McIlwain 1975). They are important components of the lipid membrane, particularly of lipid rafts. As previously described, the lipid rafts in podocytes are crucial for maintaining the slit diaphragm structure and function as signalization platforms that control the actin cytoskeleton. Moreover in recent years, bioactive sphingolipids have been discovered. These directly control the activity of intracellular enzymes and also G-coupled protein receptors, playing a major role in cell survival. In recent years, the importance of sphingolipids in kidney diseases has begun to be acknowledged. In this subchapter I will briefly review the sphingolipids and their metabolism, their main functions and finally their link with genetic and acquired glomerular diseases.

Sphingolipids and their metabolism

Sphingolipids are defined by the presence of a sphingoid base backbone, usually sphingosine. This backbone is usually linked via an amide bond to a fatty acid and to a hydrophilic head group attached to the hydroxyl on Carbon 1 (**Figure 21**). This head group may have sugar residues (glycosphingolipids) and phosphates (phosphosphingolipids) (Merrill and Carman 2015).

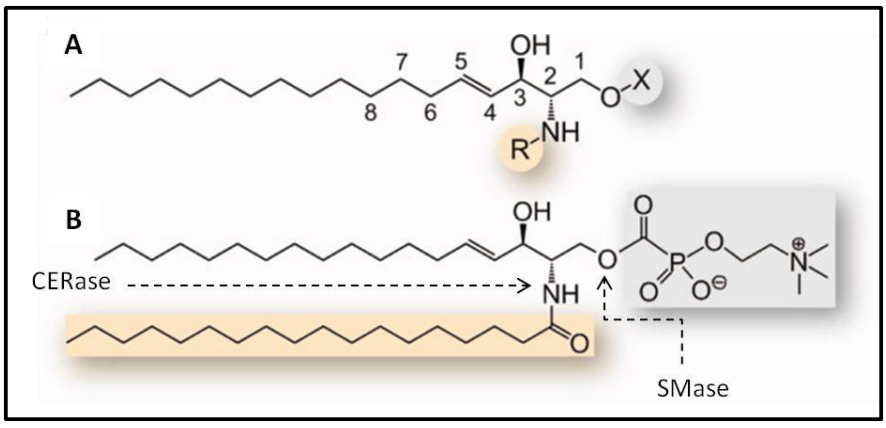

Figure 21. Chemical structure of sphingolipids. A) Sphingolipids consist of a sphingoid base backbone, generally with 18 carbons as depicted in the image. The module R is an amide linked fatty acid in sphingomyelin and ceramide, while is just an H in sphingosine. The module X is an hydrophilic region that may be in the simplest case an H, as for ceramide and sphingosine, a phosphocoline in sphingomyelin, or a phosphate in sphingosine-1-P. **B)** The sphingomyelin molecular structure is shown. Due to the action of sphingomyelinases (SMases) sphingomyelin loses its phosphocoline group, transforming in ceramide. Ceramide can be transformed in sphingosine by the action of ceramidases (CERases) that break the amide link to the fatty acid. Adapted from Bourquin *et al.* 2011.

Sphingolipids distribute asymmetrically in lipid membranes. Sphingomyelin for instance is particularly enriched in lipid rafts, together with other lipid species, such as cholesterol. As already mentioned,

lipid rafts are essential to promote protein-protein interactions and signal transduction. Sphingomyelinases at the plasma cell membrane can have an impact on the signaling properties of lipid rafts by degrading sphingomyelin in ceramides, which will subsequently lead to displacement of cholesterol at lipid rafts and alteration of fluidity of these domains (Goni and Alonso 2009, Zhang, Li et al. 2009).

Ceramides are the central sphingolipid species in the sphingolipid metabolic pathway. While three major pathways exist to synthesize ceramide there is only one catabolic pathway (**Figure 22**). The *de novo* synthesis pathway results in the generation of ceramide by condensation of serine and palmitoyl-CoA by serine palmitoyl transferase, followed by three other enzymatic steps (**Figure 22**). Ceramide can also be generated by the catabolism of sphingomyelin performed by sphingomyelinases (SMases) and producing ceramide and phosphocoline. A so-called *salvage pathway* depends on a family of enzymes known as the ceramide synthases (CerS) that couple fatty acids of different chain lengths to sphingosine, resulting in the formation of ceramide. The sphingosine used in this pathway comes from the degradation of complex sphingolipids such as glycosphingolipids and galactosylceramide.

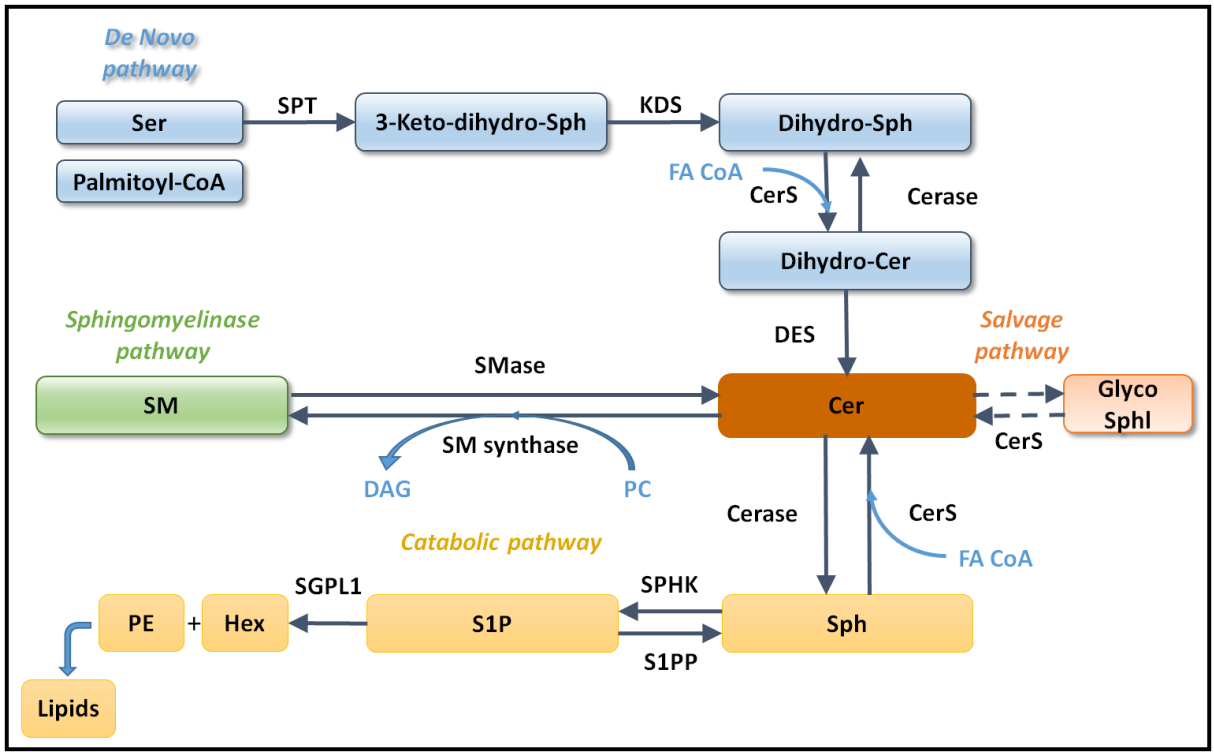

Figure 22. Overview of the sphingolipid metabolism. Ceramide (Cer) is the central molecule of the sphingolipid metabolic pathways. It can be synthesized by the *de novo*, sphingomyelinase or *salvage* pathways as depicted in the figure. The catabolic pathway of Cer begins with its transformation in sphingosine (Sph), further transformed to sphingosine-1-phosphate (S1P). S1P will finally be irreversibly degraded by S1P lyase (SGPL1) in phosphoethanolamine (PE) and hexadecenal. Ser, Serine; SPT, serine palmitoyl transferase; KDS, 3-keto-dihydro-Sph reductase; CerS, Ceramide synthase; Cerase, Ceramidases; DES, dihydro-Sph desaturase; FA CoA, fatty acyl-Coenzyme A; PC, phosphatidylcholine; DAG, diacylglycerol. S1P, Sphingosine-1-phosphate; SPHK, sphingosine kinase; S1PP, S1P phosphatase; SM, sphingomyelin; SMase, Sphingomyelinase; Glyco Sphl, Glycosphingolipids. Adapted from Bourquin *et al.* 2011.

The catabolic pathway of ceramide begins with the action of a group of enzymes called ceramidases (Cerases) that convert ceramide into sphingosine. Sphingosine can be further converted to sphingosine 1P (S1P) by sphingosine kinases (SPHKs). This important bioactive sphingolipid species is finally catabolized by sphingosine 1 phosphate lyase 1 (SGPL1), residing in the ER, to phosphoethanolamine (PE) and hexadecenal, a C16 fatty acid aldehyde. All these metabolic pathways are tightly regulated by the activities of their enzymes and their cellular compartmentalization. This allows the intracellular maintenance of physiological levels of sphingolipids (Zhou and Blom 2015).

Bioactive sphingolipids and their regulation

Besides controlling membrane fluidity and structure, some sphingolipids such as ceramides and sphingosine, or their phosphorylated counterparts' ceramide-1P or S1P, function as bioactive molecules with an impact on cell signaling and proliferation (Zhou and Blom 2015). They can act intracellularly on several direct targets or in an autocrine/paracrine way, as extracellular ligands of G-protein coupled receptors. The subcellular distribution of these bioactive sphingolipids is key to their function, as the same sphingoid base can lead to opposite effects depending on its location. To regulate this distribution cells use selective lipid transporters and compartmentalize the metabolizing enzymes to specific subcellular localizations (Zhou and Blom 2015) (**Figure 23**). The most well-known bioactive sphingolipids are ceramide and S1P, which will be described in the next section.

Ceramide and S1P – The sphingolipid rheostat

S1P and ceramide play very important roles in the regulation of cell fate. S1P is believed to promote cell survival and proliferation, while ceramide is known to induce growth arrest and cell death (Ogretmen and Hannun 2004). The opposing role of these closely related molecules is classically known as the sphingolipid rheostat (Cuvillier, Pirianov et al. 1996). The conversion of ceramide to S1P requires only two enzymatic steps, the conversion of ceramide to sphingosine by Cerases and the conversion of sphingosine to S1P by SPHKs. Therefore, the regulation of these enzymes plays a main role in cell fate (Spiegel and Milstien 2000).

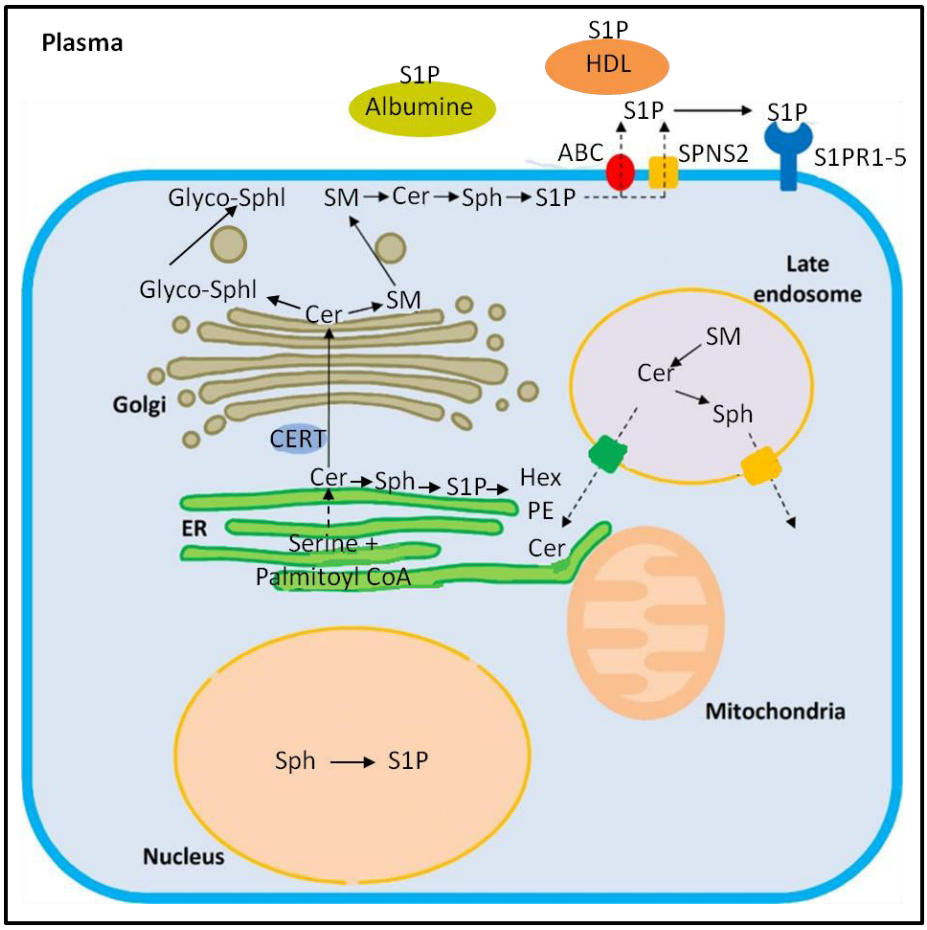

Figure 23. Cellular localization and trafficking pathways of sphingolipids. Ceramide (Cer) is synthesized *de novo* in the ER. From there it can be transported to the Golgi by Ceramide Transport Protein (CERT). Here it can be further transformed in sphingomyelin (SM) or Glyco-sphingolipids (Glyco-Sphl), that are further transported to the plasma membrane via vesicular trafficking. Late endosomes actively degrade complex sphingolipids into bioactive sphingolipid species that exit these structures by specific transporters. The endoplasmic reticulum (ER) and the mitochondria are major sites for ceramide-induced apoptosis. Sphingosine-1-phosphate (S1P), generated inside the cell can be exported to the extra-cellular space by Spinster2 (SPNS2) or ABC family transporters. S1P in plasma is bound to HDL lipoprotein and albumin. Extra-cellular S1P can bind S1P G coupled receptors (S1PR) that activate several intracellular pathways. S1P produced in the nucleus by SPHK2 can modulate HDAC proteins and transcriptional programs. S1P is irreversibly degraded in the ER into Hexadecenal (Hex) and phosphoethanolamine (PE). Adapted from Zhou *et al*. 2015.

Ceramide

The main recognized function of ceramide is as a mediator of cell senescence and cell death, both by apoptosis and autophagic mediated cell death. Following its *de novo* synthesis in the ER, ceramide can be transported by a specific transporter, ceramide transfer protein (CERT) to the Golgi for the synthesis of sphingomyelin or Ceramide 1 phosphate (Hanada, Kumagai et al. 2003) (**Figure 23**). Blocking of CERT induces ceramide accumulation on the ER inducing ER stress. High ceramide in the ER may disrupt calcium homeostasis and lead to ER stress mediated apoptosis (Bai and Pagano 1997). Ceramide may also diffuse to mitochondria (Stiban, Caputo et al. 2008) and lead to the formation of ceramide channels in the mitochondrial outer membrane (Siskind 2005) inducing

mitochondrial permeabilization, cytochrome C release and mitochondrial dependent cell death. Ceramide apoptotic cell death can also be indirectly induced by several other mechanisms. These include among others: indirect activation of BAD via the Ras/Raf cascade (Basu, Bayoumy et al. 1998), inactivation of Bcl2 protein through mitochondrial protein phosphatase 2A (PP2A) (Ruvolo, Deng et al. 1999), and growth arrest mediated by the retinoblastoma gene product (Dbaibo, Pushkareva et al. 1995), as reviewed by Mullen *et al.* (Mullen and Obeid 2012).

Moreover, ceramide can also regulate autophagy and ultimately autophagic cell death. This type of cell death is characterized by the formation of large vacuoles and early degradation of organelles, independent of apoptotic cascades (Levine and Yuan 2005). Ceramide can directly interact with LC3B-II (the lipidated form of LC3, involved in autophagy) and anchor LC3B-II autophagolysosomes to mitochondrial membranes, inducing lethal mitophagy (Sentelle, Senkal et al. 2012). Moreover ceramide can induce autophagic death by activating the transcription of the death-inducing mitochondrial protein BNIP3, through an unknown mechanism (Daido, Kanzawa et al. 2004).

In line with the toxic effects of ceramides, cancer cells usually maintain ceramides at low levels by increasing the expression of enzymes from the catabolic pathway, such as the Cerases (Shah, Zhang et al. 2008).

S1P intra-cellular effects

As mentioned, S1P can be generated by SPHKs. Moreover, S1P levels can be further regulated by their dephosphorylation back into sphingosine by the S1P phosphatases, as well as by irreversible degradation mediated by SGPL1. Interestingly, there are two isoforms of SPHK, SPHK1 and SPHK2. Although sharing similar domains, these enzymes have different tissue and subcellular distributions as well as specific developmental expression patterns (Kohama, Olivera et al. 1998, Liu, Sugiura et al. 2000).

SPHK1 resides in the cytoplasm and is activated by several extracellular growth and survival stimuli, regulating autophagy in response to nutrients (Lanterman and Saba 1998, Lavieu, Scarlatti et al. 2006). However, the exact mechanism of autophagy regulation as well as whether S1P signaling acts through or parallel to the mammalian target of rapamycin (mTOR) pathway is not clear (Harvald, Olsen et al. 2015). According to its roles in growth and survival, SPHK1 is able to induce DNA synthesis and cell proliferation, as well as resistance to serum deprivation and to ceramide-induced apoptosis (Olivera, Kohama et al. 1999). Moreover, S1P produced by SPHK1 can promote survival by modulating calcium mobilization from intracellular stores, activation of the MAPK pathway or

through the extracellular signal-regulated kinase (ERK) as well inhibition of c-Jun-N-terminal protein kinase (c-JUNK) (Mattie, Brooker et al. 1994, Wu, Spiegel et al. 1995, Cuvillier, Pirianov et al. 1996, Meyer zu Heringdorf, Lass et al. 1998).

Conversely, SPHK2 is predominantly found in the nucleus, with both nuclear localization and export signals allowing for active regulation of its localization (Igarashi, Okada et al. 2003, Ding, Sonoda et al. 2007). These localization signals are not present in SPHK1. In a diametrically opposed function to SPHK1, SPHK2 inhibits cell growth and is pro-apoptotic. Nuclear SPHK2 prevents DNA synthesis (Igarashi, Okada et al. 2003) and can regulate gene expression through indirect regulation of histones deacetylases via S1P (Hait, Allegood et al. 2009). SPHK2 induced apoptosis seems to be partially dependent on the increased conversion of sphingosine to ceramide by an unknown regulatory mechanism and results in calcium transport to the mitochondria and cytochrome C release (Maceyka, Sankala et al. 2005). Moreover SPHK2 has a Bcl-2 homology-3 (BH3) domain that may displace Beclin1 from the Beclin1/Bcl-2 complex in order to induce apoptosis (Sheng, Zhang et al. 2014).

Interestingly mice KO for either *Sphk1* or *Sphk2* develop normally and do not show any obvious abnormalities, although they have reduced SPHK activity (Allende, Sasaki et al. 2004, Mizugishi, Yamashita et al. 2005). However the double KO of *Sphk1* and *Sphk2* leads to embryonic lethality with severe defects of neurogenesis and angiogenesis (Mizugishi, Yamashita et al. 2005). This suggests that the two enzymes can partially compensate for one another's loss during development.

Through the regulation of intracellular S1P and ceramides, SGPL1 can modulate intracellular mechanisms, including but not limited to proliferation and apoptosis. However the exact mechanisms have not been completely elucidated. *Sgpl1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) show alterations in intracellular calcium homeostasis (Claas, ter Braak et al. 2010), accumulate intracellular S1P mainly in cell nuclei and show decreased expression and activity of the histone deacetylases HDAC1 and 3 (Ihlefeld, Claas et al. 2012), suggesting that S1P can also induce changes in transcriptional programs.

Extracellular signaling of S1P

Besides its intracellular functions S1P can be exported out of the cell by two specific types of transporters, spinster 2 (SPNS2) and ATP-binding cassette (ABC) transporters, such as ABCB1, ABCA7 and ABCC1 (Nishi, Kobayashi et al. 2014) (**Figure 23)**. Erythrocytes, platelets and endothelial cells release high amounts of S1P into the plasma (Pappu, Schwab et al. 2007, Venkataraman, Lee et al.

2008, Fukuhara, Simmons et al. 2012, Hisano, Kobayashi et al. 2012), where it binds to several protein carriers, such as albumin (≈30% of S1P) and HDL (≈60%), presumably contributing to its stability (Argraves and Argraves 2007). However, in tissues, S1P concentration is maintained at low levels due to high S1P-degrading activity conferred by SGPL1. In physiological conditions the levels of S1P in plasma or lymph are thus much higher than in tissues (Olivera, Allende et al. 2013).

Extracellular S1P may act as a specific ligand for a family of five G protein coupled receptors, named S1P receptors (S1PRs) (Chun, Goetzl et al. 2002) (**Figure 23**). From the five S1PRs, S1PR1-3 are rather ubiquitous, S1PR1 being one of the most highly expressed (Regard, Sato et al. 2008). Recently, a genetically engineered mouse that turns on a GFP reporter gene once S1PR1 is activated has provided further insight into S1PR1 signaling (Kono, Tucker et al. 2014). In embryos, S1PR1 activation was higher in the nervous and cardiovascular systems; concordant with these finding *S1pr1* KO mice show defects in vascular and neuronal development (Liu, Wada et al. 2000, Mizugishi, Yamashita et al. 2005). In adults, S1PR1 activation was mostly restricted to the endothelial cells, especially those of lymphoid tissues; however, under lipopolysaccharide (LPS) induced inflammation, S1PR1 activity was highly increased in the endothelium and also appeared in parenchymal cells such as the hepatocytes. This was shown to be due to hematopoietically derived S1P that possibly accumulated into the interstitial tissue through vascular leakage (Kono, Tucker et al. 2014). In fact, tissue levels of S1P have been shown to be increased in several inflammatory diseases, as reviewed by Gomez-Munoz *et al.* (Gomez-Munoz, Presa et al. 2016). However, the consequences of interstitial S1P and S1PR signaling on the reparative process or fibrotic response are not well understood (Proia and Hla 2015).

The other S1PRs are less well known, but S1PR2 and S1PR3 also seem to participate in the angiogenic process. In fact, single *S1pr2* or *S1pr3* KO mice do not have major abnormalities and these mice are viable and fertile, however, the double KO leads to vascular defects during development and partial embryonic lethality (Kono, Mi et al. 2004), in favor of a cooperative function during development.

S1P signaling through S1PRs induces several cellular processes, such as cytoskeletal rearrangements and cell migration by modulating RhoGTPase activity (Wang, Van Brocklyn et al. 1999, Okamoto, Takuwa et al. 2000, Garcia, Liu et al. 2001, Lee, Thangada et al. 2001). However, several other functions have also been ascribed to S1PR signaling, such as the ability to regulate cell survival and proliferation via activation of several key protein kinases such as Akt and ERK (Hla, Lee et al. 2001). Adding complexity to S1PR signaling, each receptor seems to have distinctive signaling pathways that differ between cell type and that in some cases can elicit antagonistic cellular responses (Blaho and Hla 2014).

A well-recognized function of extracellular S1P is the ability to modulate hematopoietic cell

trafficking from tissues to plasma (Brinkmann, Davis et al. 2002, Mandala, Hajdu et al. 2002). S1PR1 is known to be expressed in lymphocytes and to mediate their migration from the lymphoid tissues to the blood or lymph, in response to the S1P gradient (low in tissues and high in blood/lymph) (Cyster and Schwab 2012). Egress of hematopoietic stem and progenitor cells from the bone marrow into the blood is also mediated by S1PR1, toward high S1P concentrations (Juarez, Harun et al. 2012). Consequently, disruption of the S1P tissue-blood gradient leads to lymphopenia. This was observed in mice that lack the S1P transporter SPNS2, responsible for the majority of S1P secretion into the circulation (Fukuhara, Simmons et al. 2012, Nagahashi, Kim et al. 2013), or with the KO or the pharmacological inhibition of SGPL1 in mice, that show greatly increased tissue levels of S1P (Schwab, Pereira et al. 2005, Vogel, Donoviel et al. 2009, Allende, Bektas et al. 2011) .

In blood, these cells are exposed to high S1P concentration. This leads to the down-regulation of S1PRs through GPCR-kinase 2 mediated phosphorylation and internalization (Thangada, Khanna et al. 2010, Arnon, Xu et al. 2011). Through the downregulation of S1PRs, these cells can undergo shear resistant adhesion and migrate back across the endothelium (Arnon, Xu et al. 2011).

Interestingly, S1P signals also serve for lymphocyte migration and positioning within tissues (Arnon, Horton et al. 2013). Fingolimod (or FTY720), a sphingosine analog that is phosphorylated through SPHK2 to produce FTY720-phosphate, functions as a S1PR1 functional antagonist. In fact, FTY720-phosphate down-regulates S1PR1 from the cell surface, blocking the egress of lymphocytes from lymphoid tissues into the circulation (Brinkmann, Billich et al. 2010). Due to its effect on lymphocyte trafficking Fingolimod is now approved for the treatment of auto-immune inflammatory diseases such as multiple sclerosis.

Sphingolipids in podocyte biology

Genetic deficiencies in the sphingolipid metabolic pathway can give rise to the toxic accumulation of sphingolipid byproducts in cellular organelles and membranes. These form a class of inherited diseases called sphingolipidoses that include Niemman-Pick disease (sphingomyelin and cholesterol accumulation), Tay-Sachs disease and Sandhoff disease (GM2 ganglioside accumulation), Gaucher's disease (glucocerebroside accumulation) and Fabry's disease (glycolipid accumulation, namely globotriaosylceramide). Most of these diseases display a neurological phenotype, suggesting that neurons are particularly sensitive to changes in sphingolipid metabolism. The increased sensibility of neuronal cells to defective sphingolipid metabolism has been suggested to be related to defective lipid raft signaling (Sural-Fehr and Bongarzone 2016).

Interestingly, some sphingolipidoses also exhibit a kidney defect. In fact, in Fabry disease, where alpha-galactosidase A (GLA) is deficient, the kidney, brain and heart, are the predominantly affected organs. Accumulation of globotriaosylceramide (Gb3) has been observed in lysosomes, the ER and nucleus of renal cells (Askari, Kaneski et al. 2007). Podocytes appear hypertrophic, with multiple vacuoles giving a foamy appearance, and characteristic inclusion bodies of glycolipids called "myelin figures" or "Zebra bodies". Mesangial expansion can also be seen (Alroy, Sabnis et al. 2002). These findings are associated with progressive podocyte injury, foot process effacement, podocyturia and proteinuria (Thurberg, Rennke et al. 2002, Najafian, Svarstad et al. 2011, Trimarchi, Canzonieri et al. 2016). Therapy with recombinant human GLA has been shown to attenuate the histological findings and slow the loss of kidney function (Thurberg, Rennke et al. 2002, Quinta, Rodrigues et al. 2014). Although the exact pathological mechanism is not completely understood a link to defective autophagy has been suggested in a study performed in cultured human podocytes. In this study silencing of GLA lead to Gb3 accumulation and decreased mTOR activity together with dysregulated autophagy (Liebau, Braun et al. 2013). Gb3 was also shown to induce the expression of inflammatory molecules and fibrosis mediators in cultured human podocytes (Sanchez-Nino, Sanz et al. 2011, Sanchez-Nino, Carpio et al. 2015).

Niemman-Pick type A and B are caused by mutations in *SMPD1*, encoding an acid sphingomyelinase (ASMase) and leading to accumulation of sphingomyelin. Characteristically there is neurological involvement and deposition of lipid laden macrophages in bone marrow, liver and kidney in these patients and in *Smpd1*-/- mice (Briere, Calman et al. 1976, Kuemmel, Thiele et al. 1997). Recently a study reported that sphingomyelin overload disturbs the maturation and closure of autophagic membranes and induces a traffic defect in the autophagy related protein 9A (ATG9A) (Corcelle-Termeau, Vindelov et al. 2016). Moreover cholesterol accumulation in the plasma membrane with disruption of lipid raft structures has been shown to cause impaired insulin signaling (Vainio, Bykov et al. 2005).

The presence of kidney defects has been more rarely described in other sphingolipidoses, such as Gaucher disease (Chander, Nurse et al. 1979), Tay Sachs and Sandhoff diseases (Sandhoff, Andreae et al. 1968, Tatematsu, Imaida et al. 1981).

Interestingly genetic diseases other than sphingolipidoses can also lead to a disturbance of the sphingolipid content in podocytes. In fact, mutations in *SCARB2*, encoding for the ubiquitously expressed lysosomal integral membrane protein type 2 (LIMP2) are associated with epilepsy, demyelinating peripheral neuropathy and SRNS with FSGS lesions (Berkovic, Dibbens et al. 2008), a syndrome also called action myoclonus-renal failure syndrome. Although the function of LIMP2 is not

completely known, it is thought to play a role in the biogenesis and maintenance of endosomal and lysosomal compartments and also acts as a receptor for the lysosomal delivery of acid hydrolase β-glucocerebrosidase (Reczek, Schwake et al. 2007). Its dysfunction results in the accumulation of glucosylceramide and other glycosphingolipids (Gaspar, Kallemeijn et al. 2014, Zigdon, Meshcheriakova et al. 2017). The FSGS phenotype is not 100% penetrant and some cases only exhibit tubular proteinuria, that has been linked to lysosomal dysfunction and subsequent perturbation of the degradation of reabsorbed proteins in proximal tubular cells (Desmond, Lee et al. 2011, Lee, Gleich et al. 2013). To date, the precise mechanism leading to the characteristic podocytopathy of this disease is not known.

Few studies have tried to address the role of several sphingolipid species on podocyte functions. i.e. it was shown that sFLT, a soluble VEGF receptor, binds to the ganglioside GM3 in lipid rafts at the surface of podocytes. sFLT binding to GM3 promotes podocyte adhesion and actin reorganization (Jin, Sison et al. 2012). Interestingly sFLT is increased during pre-eclampsia, a disorder characterized by high blood pressure and proteinuria in the third trimester of pregnancy, generalized endothelial dysfunction and podocyte injury. Another example relates to the podocyte specific O-acetylated GD3 ganglioside that also localizes to lipid rafts. Treatment with an antibody against this ganglioside leads to foot process effacement and nephrin phosphorylation and displacement from the slit diaphragm (Dekan, Miettinen et al. 1990, Simons, Schwarz et al. 2001). Interestingly, in the PAN model of FSGS a reduction in O-acetylated GD3 ganglioside has been described (Holthofer, Reivinen et al. 1996). These findings suggest that like in neurons, sphingolipid content in the lipid rafts of podocytes is important for the maintenance of a functional slit diaphragm and the activation of cell signaling pathways.

More recently, an important role for acid sphingomyelinase-like phosphodiesterase 3b (SMPDL-3b), was shown in FSGS and diabetic kidney disease (Fornoni, Sageshima et al. 2011, Yoo, Pedigo et al. 2015). SMPDL3b is localized to podocyte lipid rafts, and although its exact enzymatic function remains unknown, it is thought to convert sphingomyelin to ceramide, like its homolog enzyme acid sphingomyelinase (ASMase) (Fornoni, Sageshima et al. 2011). It was found that in biopsies of transplanted kidneys with FSGS relapse, the number of SMPDL3b and ASMase positive podocytes was reduced. Moreover, in cultured podocytes exposed to sera from patients with recurrent FSGS, a decrease in ASMase activity and SMPDL-3b down-regulation was observed, together with disruption of the actin cytoskeleton. Overexpression of SMPDL-3b or treatment with rituximab, an anti-CD20 antibody also known to stabilize SMPDL-3b (Perosa, Favoino et al. 2006, Tasaki, Shimizu et al. 2014), was able to prevent the cytoskeletal disorganization and podocyte apoptosis induced by patient sera (Fornoni, Sageshima et al. 2011). Another group showed that radiation-induced podocytopathy in

mice also leads to down-regulation of SMPDL-3b. However, unlike in FSGS or radiation-induced podocyte lesions, in diabetic kidney disease the levels of SMPDL-3b were increased in glomeruli (Yoo, Pedigo et al. 2015). Moreover pharmacological inhibition of ASMase reduced proteinuria in diabetic kidney disease but not in FSGS (Yoo, Pedigo et al. 2015). This suggests that the expression of SMPDL-3b changes according to the type of glomerular disease and it remains difficult to assess its causality. Although it has been suggested that these modulations may be dependent on modification of lipid rafts, SMPDL-3b's relationship to ceramide and S1P is less well explored and the mechanism remains unknown.

Another interesting finding is that SUPAR, a suggested permeability factor, induces podocyte defects through lipid mediated activation of β3 integrin signaling, as sequestration of plasma membrane lipids with cyclodextrine prevents SUPAR from exerting its effect on podocytes (Wei, Moller et al. 2008).

Regarding the effect of the bioactive lipid S1P in the kidney, literature is also scarce, especially for the specific effect of S1P in podocytes. In the kidney several S1PRs are expressed differentially between different cell types, i.e. podocytes express S1PR1-4, but not S1PR5 (Awad, Rouse et al. 2011) and mesangial cells express S1PR1-3 and S1PR5 but not S1PR4 (Koch, Volzke et al. 2013). Moreover, several growth factors implicated in the pathogenesis of glomerular diseases have been shown to modulate the levels of S1P, such as PDGF, VEGF and angiogenic growth factor (Merscher and Fornoni 2014). Not surprisingly, S1P is increased in inflammatory glomerulopathies (Sun, Wang et al. 2017) and has been shown to promote fibrosis in models of kidney disease that are partially moderated by inflammatory mediators (Thangada, Shapiro et al. 2014). However, an inflammation-independent deleterious role for S1P has also been suggested (Shiohira, Yoshida et al. 2013). Interestingly, also in the kidney, SPHK1 and SPHK2 play differential roles. In response to acute kidney injury, *Sphk2* KO mice show reduced fibrosis (Park, Kim et al. 2011, Bajwa, Huang et al. 2017) while *Sphk1* KO mice have more severe lesions (Park, Kim et al. 2011) and specific overexpression of SPHK1 seems to be renoprotective (Park, Kim et al. 2011).

Epigenetic regulation and podocyte homeostasis

Epigenetics is widely defined as heritable changes in the genome that occur without alterations to the DNA sequence itself (Bonasio, Tu et al. 2010). This includes mitotically and/or meiotically heritable changes. Epigenetic information plays an essential role in various biological processes including embryonic development, cell differentiation, adult cell renewal and ageing. Furthermore, as external physiological stimuli can lead to persistent changes in the chromatin state, epigenetic markers also function as key mediators of the environmental influence on phenotype. Deregulation of epigenetic control has been implied in many human diseases, including cancer (Baylin and Ohm 2006), autoimmune diseases, neurological disorders (Portela and Esteller 2010), Diabetes mellitus (Villeneuve and Natarajan 2010), cardiovascular (Ordovas and Smith 2010) and kidney diseases (Ekstrom and Stenvinkel 2009, Mohtat and Susztak 2010, Dwivedi, Herman et al. 2011, Elie, Fakhoury et al. 2011, Liakopoulos, Georgianos et al. 2011). In this section, I will briefly review the major mechanisms of epigenetic regulation and review the impact of epigenetic mechanisms on podocyte biology and podocytopathies.

Epigenetic mechanisms

In general epigenetic states are established by the transient expression/activation of transcription factors that respond to diverse stimuli, such as environmental or developmental cues. The exact mechanisms by which transcription factors achieve this epigenetic regulation are not completely understood but may include the direct recruitment of proteins from the epigenetic machinery to a specific genomic region, or the transcription of non-coding RNAs or proteins that will then help to establish a specific epigenetic marker (Bonasio, Tu et al. 2010). The major epigenetic mechanisms include DNA methylation, histone modifications and nucleosome remodelling. Non-coding RNAs can also be broadly classified as epigenetic markers but these will not be further detailed in this thesis.

DNA methylation

DNA methylation was the first epigenetic mechanism described (Holliday and Pugh 1975) and is the most widely studied epigenetic modification in humans. DNA methylation is involved in gene dosage reduction mechanisms such as genomic imprinting (Kacem and Feil 2009) and X chromosome inactivation (Reik and Lewis 2005). DNA methylation is achieved by DNA methyltransferases (DNMT)

that are classified as either *de novo* DNMTs (DNMT 3A and 3B) or maintenance DNMTs (DNMT1). Methylation occurs predominantly in the context of CpG dinucleotides, which cluster in regions that are called CpG islands. In general, CpG island methylation in promoter regions is associated with gene silencing (Ushijima 2005) through the recruitment of methyl-CpG binding domain (MBD) proteins (Lopez-Serra, Ballestar et al. 2006) that interact with histone modifiers and chromatin remodelling complexes to mediate transcriptional repression (Lopez-Serra and Esteller 2008). During development and in differentiated tissues, CpG islands become methylated resulting in gene silencing in a tissue-specific manner (Straussman, Nejman et al. 2009). Furthermore, in the context of disease, CpG promoter methylation can occur at key genes whose silencing contributes to the pathogenic process (Portela and Esteller 2010).

Histone modifications

The DNA in chromatin is packaged around histone proteins in units that are called nucleosomes. Each nucleosome is composed of 147 bp of DNA wrapped around an octameric core of histone proteins containing, in duplicate, four core histones: H2A, H2B, H3, and H4. Nucleosomes are spaced by a linker region of approximately 50 bp that associates with the H1 linker histone. Each core histone is composed of a structured globular domain and an N-terminal tail (**Figure 24**). Histone tails can suffer multiple post translational modifications (PTMs), including phosphorylation, ubiquitination, acetylation, and methylation (Martin and Zhang 2005, Kouzarides 2007), among others. Different combinations of histone modifications, named the "histone code", participate in the regulation of chromatin structure and transcriptional status (Strahl and Allis 2000, Jenuwein and Allis 2001, Henikoff 2008).

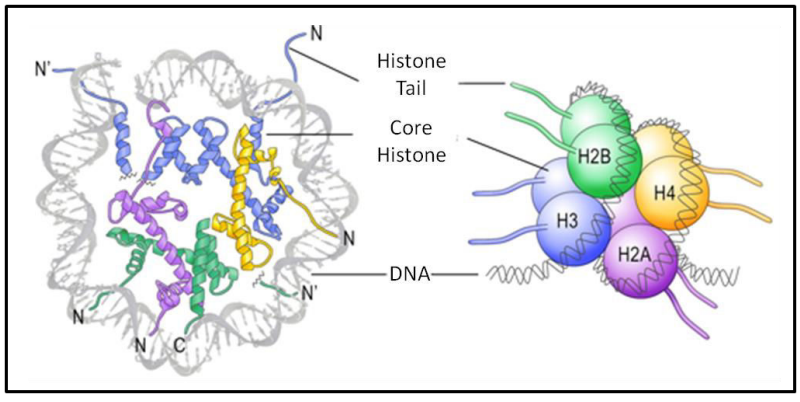

Figure 24. Nucleosome structure. Each nucleosome is composed of two molecules of each H2A, H2B, H3 and H4 core histones. DNA raps around this core. Each histone project their N-terminal histone tails outside of the hydrophobic core, allowing the occurrence of post-translational modifications. Adapted from Graff *et al.* 2008.

There are several enzymes that catalyze PTMs as well as others who reset these modifications, conferring a reversibility characteristic to most of the epigenetic markers (Kouzarides 2007). Of the enzymes that modify histones, methyltransferases, histone demethylases and kinases are the most

specific to individual histone subunits and residues (Kouzarides 2007). Methylation of specific histone residues marks gene activation or silencing. For instance, methylation of arginine residues in histones is associated with transcriptional activation (Chen, Ma et al. 1999), whereas histone lysine methylation drives gene activation or silencing depending on the residue modified and number of methylations (Turner 2002). Polycomb and Trithorax gene families are important regulators of gene repression and activation, respectively, through several mechanisms that include recognition and modulation of histone methylation (Schwartz and Pirrotta 2007, Poynter and Kadoch 2016). Histone methylation modifications are often persistent through mitosis in a cellular lineage, leading to "long term memory" and have received much attention in recent years.

On the other hand, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are not highly specific and can modify several residues and also many proteins other than histones. For this reason HATs and HDACs are also called lysine acetyl-transferases (KATs) and lysine deacetylases (KDACs). Most HDACs function to reset chromatin by removing acetylation at active genes, leading to a closed chromatin structure and transcriptional inactivation. HATs, instead, are mainly linked to open chromatin structure and transcriptional activation (Wang, Zang et al. 2009) (**Figure 25**). These modifications can be dynamic and be induced rapidly in response to injury.

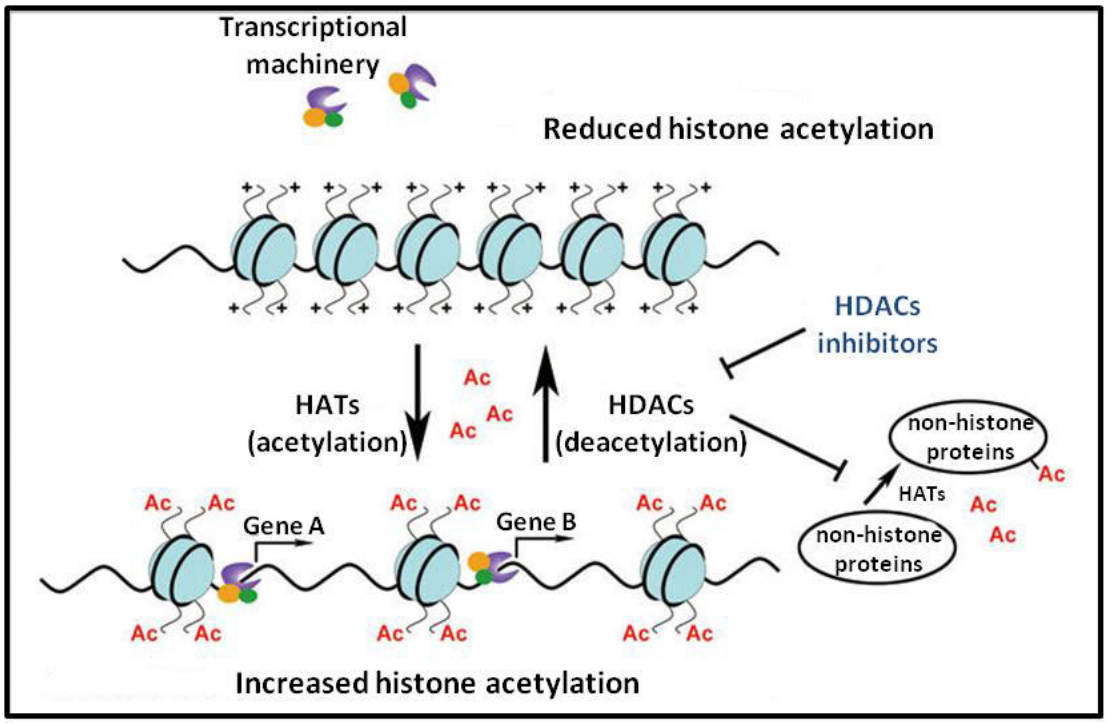

Figure 25. Lysine acetylation is balanced by HATs and HDACs. Lysine residues from protruding histone tails are acetylated by HATs leading to modification of the chromatin structure and facilitating the accessibility of the transcriptional machinery to promoter regions and the activation of gene expression. HDACs perform the inverse action. The acetylation state and functions of many non-histone proteins are also controlled by HATs and HDACs. Adapted from Wang *et al.* 2014.

HDACs are grouped into four classes of proteins, based on their homology to yeast HDACs: class I (HDAC1-3, HDAC8), class II (HDAC4-7, HDAC9-10), class III (sirtuins - SIRT1-7), and class IV (HDAC11). HATs on the other hand, can be classified according to their cellular localisation in type A HATs, that are nuclear, and type B HATs, that exist in the cytoplasm and acetylate newly formed histones. Based on their sequence homology, nuclear HATs are further grouped into five families, the three major ones being the Gcn5-related N-acetyltransferases (GNAT), p300 and CREB binding protein (p300/CBP) and Moz, Ybf2/Sas3, Sas2, Tip60 (MYST) (**Table 2**).

Table 2. Nuclear histone acetyltransferases: families, subtypes, and alternative nomenclature

Family	Subtype	Other names frequently used
GNAT	KAT2A	Gcn5
	KAT2B	PCAF
MYST	KAT5	TIP60
	KAT6A	MOZ, MYST3
	KAT6B	MORF, MYST4
	KAT7	HBO1, MYST2
	KAT8	MOF, MYST1
p300/CBP	KAT3B	p300
	KAT3A	CBP
Transcription co-activators	KAT4	TAF1, TBP
	KAT12	TIFIIIC90
Steroid receptor co-activators	KAT13A	SRC1
	KAT13B	SCR3, AIB1, ACTR
	KAT13C	p600
	KAT13D	CLOCK

Nucleosomal remodelling

Nucleosome positioning determines accessibility of the transcription factors to their target DNA sequence. Several groups of large macromolecular complexes are known to move, destabilize or restructure nucleosomes in an ATP-dependent manner. These complexes, known as chromatin remodelling complexes, can be classified into four families (SWI/SNF, ISWI, CHD and INO80) that share similar ATPase domains but differ in the composition of their unique subunits (Ho and Crabtree 2010).

Epigenetic regulation in podocytes

Epigenetic changes are particularly important for defining the cell phenotype upon development and for maintaining cell identity in regenerative responses to injury. Although mature podocytes are terminally differentiated and non-dividing cells, several studies show that they depend on the epigenetic machinery in order to maintain a stable gene expression pattern. In the majority of cases the crucial role of the epigenetic actors is seen in conditions where podocytes are exposed to noxious stimuli or during aging. This was nicely shown by Lefevre et al. who performed the mouse podocyte specific KO of the gene *Paxip1*, encoding PTIP, an essential component of the histone H3 lysine 4 (H3K4) methyltransferase complex. This led to podocyte foot process effacement and proteinuria, manifesting in mice at 3 months of age and aggravating with age. Chromatin immunoprecipitation (ChIP) showed lower H3K4 methylation at the *Ntrk3* (neurotrophic tyrosine kinase receptor, type 3) locus, whose expression was significantly reduced and whose function may be essential for podocyte foot process patterning (Lefevre, Patel et al. 2010). In another study, inhibition of the histone methyltransferase EZH2 (enzyme enhancer of zeste homolog 2), a subunit of the Polycomb Repressive Complex, with a pharmacological compound (S-adenosylhomocysteine hydrolase inhibitor 3-deazaneplanocin A (DZNep)) or by RNA interference, rendered cultured podocytes vulnerable to high glucose concentrations, leading to podocyte cell death and augmented oxidative stress. *In vivo* administration of DZNep to mice led to proteinuria in the metabolic adverse conditions provided by the diabetic *milieu* (Siddiqi, Majumder et al. 2016). By using ChIP sequencing and *in silico* analysis in cultured podocytes and diabetic rodents, these authors further showed that inhibition of EZH2 led to decreased repression of the transcription factor Pax6 and transcriptional activation of the antioxidant inhibitor *TxnIP*.

Interestingly, KAT8, also called MYST1, a major H4K16 acetyltransferase that plays a crucial role in proliferating tissues and embryonic development, is also dispensable for mature kidney podocytes in physiological conditions (Sheikh, Bechtel-Walz et al. 2016). However, in response to injury it plays a main role in podocyte adaption and survival *in vivo*. In fact, when exposed to adriamycin, *Kat8*$^{-/-}$ podocytes show chromatin condensation defects and altered endoplamic reticulum and Golgi structure. Mice also develop glomerulosclerosis, significant proteinuria and decreased kidney function compared to controls. These authors further show that KAT8 binds to promoters of genes encoding endo-lysossome pathways, known to be important for podocyte function (Sheikh, Bechtel-Walz et al. 2016).

Interference with HDAC activity also leads to modulation of the podocyte response to injury in several models of glomerulopathy. Administration of valproic acid, a known inhibitor of HDAC classes

I and II, had a protective effect on adriamycin-induced podocyte injury in mice, preventing podocyte foot process effacement and detachment as well as the glomerulosclerosis process (Van Beneden, Geers et al. 2011). Also, in diabetic nephropathy, several studies have highlighted the beneficial effect of class I, II and IV HDAC pharmacological inhibition (Advani, Huang et al. 2011, Gilbert, Huang et al. 2011). In the same context, HDACs have been shown to affect not only epigenetic mechanisms but also to regulate the activity of other proteins, such as p65 and STAT1 transcription factors, controlling inflammation and autophagy, or cortactin, with an impact on the actin cytoskeleton (Wang, Liu et al. 2014, Nakatani and Inagi 2016). In an experimental model of lupus nephritis a significant decrease of renal pathology scores and proteinuria was observed with the broad-spectrum HDAC class I and II inhibitor, trichostatin A (Mishra, Reilly et al. 2003). Likewise, HDAC class I and II inhibitors were found to reduce proteinuria, glomerulosclerosis and interstitial fibrosis on a model of anti-GBM glomerulonephritis (Imai, Hishikawa et al. 2007) and to reduce proteinuria in a model of mesangial proliferative glomerulonephritis (Freidkin, Herman et al. 2010).

On the other hand, class III HDACs, such as Sirtuin1, seem to be renoprotective (Hasegawa, Wakino et al. 2013, Nakatani and Inagi 2016). Sirtuins have been shown to exert a beneficial effect on telomere length maintenance (Palacios, Herranz et al. 2010) and to promote autophagy and cell survival (Kume, Uzu et al. 2010). Accordingly, decrease of SIRTs has been associated with kidney ageing and mitochondrial damage (Kume, Uzu et al. 2010).

During development, epigenetic mechanisms are highly active, and some of the epigenetic actors are involved in podocyte maturation. This is the case for the chromodomain helicase DNA binding protein 2 (CHD2), a member of the nucleosome chromatin remodelling complexes. *Chd2* heterozygous-mutant mice develop glomerulopathy, proteinuria and significantly impaired kidney function (Marfella, Henninger et al. 2008) while homozygous mutants show reduced growth and perinatal lethality (Marfella, Ohkawa et al. 2006).

Another example of the importance of epigenetics in podocytes comes from human genetics. In humans *SMARCAL1* mutations cause the autosomal recessive syndrome Schimke Immuno-Osseous Dysplasia (SIOD), characterized by SRNS, skeletal dysplasia, T cell immunodeficiency and arteriosclerosis leading to strokes (Boerkoel, Takashima et al. 2002). These can be either nonsense or missense loss of function mutations (Elizondo, Cho et al. 2009). *SMARCAL1* encodes the SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, subfamily A-Like 1 (SMARCAL1) that belongs to the chromatin remodeling SNF2 family of helicases. SMARCAL1 contains a replication protein A (RPA) interacting motif, two HARP domains, and a helicase domain with ATPase activity at its C terminus (Lugli, Sotiriou et al. 2017). SMARCAL is an annealing helicase that can bind and anneal

complementary single strand DNA through its interaction with RPA (Yusufzai and Kadonaga 2008). It is known to be specifically recruited to sites of DNA damage and to stabilize and restore damaged replication forks (Postow, Woo et al. 2009, Betous, Glick et al. 2013). SMARCAL1 is also involved in alternative lengthening of telomeres, protecting them from DNA damage (Poole, Zhao et al. 2015, Cox, Marechal et al. 2016). Moreover, it directly regulates gene expression by binding and possibly altering the chromatin conformation of gene promoter regions, as it is the case for the *c-Myc* gene (Sharma, Bansal et al. 2015). In *Drosophila* the SMARCAL homolog has been shown to induce histone modifications and gene expression changes through the Polycomb Group and Trithorax Group complexes (Morimoto, Choi et al. 2016). In patients, chromosomal instability has been documented (Simon, Lev et al. 2014). Moreover SMARCAL1 was also shown to genetically interact with Notch and Wnt pathways in *Drosophila* and expression of their targets were also dysregulated in patient kidney biopsies, raising one possible pathogenic mechanism for the glomerular disease (Morimoto, Myung et al. 2016). Interestingly, the *Smarcal1* KO mice do not have an overt phenotype; however they are hypersensitive to genotoxic stress, developing part of the SCIOD phenotype when exposed to genotoxic agents (Baradaran-Heravi, Raams et al. 2012). In contrast, the zebrafish morpholino-mediated KD of the *SMARCAL* homolog has a spontaneous phenotype that phenocopies that of patients (Huang, Gu et al. 2010).

Results and Discussion

Part I

A new syndromic form of SRNS caused by *SGPL1* mutations

Article: Mutations in sphingosine-1-phosphate lyase cause nephrosis with ichthyosis and adrenal insufficiency

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Mutations in sphingosine-1-phosphate lyase cause nephrosis with ichthyosis and adrenal insufficiency

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Steroid-resistant nephrotic syndrome (SRNS) causes 15% of chronic kidney disease cases. A mutation in 1 of over 40 monogenic genes can be detected in approximately 30% of individuals with SRNS whose symptoms manifest before 25 years of age. However, in many patients, the genetic etiology remains unknown. Here, we have performed whole exome sequencing to identify recessive causes of SRNS. In 7 families with SRNS and facultative ichthyosis, adrenal insufficiency, immunodeficiency, and neurological defects, we identified 9 different recessive mutations in *SGPL1*, which encodes sphingosine-1-phosphate (S1P) lyase. All mutations resulted in reduced or absent SGPL1 protein and/or enzyme activity. Overexpression of cDNA representing *SGPL1* mutations resulted in subcellular mislocalization of SGPL1. Furthermore, expression of WT human SGPL1 rescued growth of SGPL1-deficient *dpl1*Δ yeast strains, whereas expression of disease-associated variants did not. Immunofluorescence revealed SGPL1 expression in mouse podocytes and mesangial cells. Knockdown of *Sgpl1* in rat mesangial cells inhibited cell migration, which was partially rescued by VPC23109, an S1P receptor antagonist. In *Drosophila*, *Sply* mutants, which lack SGPL1, displayed a phenotype reminiscent of nephrotic syndrome in nephrocytes. WT *Sply*, but not the disease-associated variants, rescued this phenotype. Together, these results indicate that *SGPL1* mutations cause a syndromic form of SRNS.

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Introduction

Steroid-resistant nephrotic syndrome (SRNS), when also resistant to other immunosuppressive agents, leads to chronic kidney disease (CKD) within a few years of onset, requiring renal replacement therapy for survival. It causes 15% of all end-stage kidney disease that manifests by 25 years of age (1). Histologically, SRNS manifests mostly as focal segmental glomerulosclerosis (FSGS) (2).

The first insights into the pathogenesis of SRNS were gained by the discovery of monogenic causes of SRNS, revealing that the encoded proteins are essential for the function of the renal glomerular cells called podocytes (3, 4). We recently demonstrated in a worldwide cohort of 1,783 families that a monogenic cause of SRNS can be detected in 1 of 27 genes in approximately 30% of SRNS cases manifesting before age 25 years (5). Currently, more than 40 monogenic forms of SRNS have been identified (6).

Interestingly, syndromic forms of SRNS have been increasingly characterized and display variable involvement of other organs besides the kidney, in most of the cases the central nervous system, but also the genital tract, eye, muscle, bone, and the immune system. To date, mutations have been identified in transcription factors and nuclear proteins (WT1, LMX1B, SMARCAL1, NXF5, NUP93, NUP205, and XPO5), lysosomal proteins (SCARB2), basement membrane proteins (LAMB2), and proteins involved in COQ_{10} biosynthesis (COQ2, COQ6, PDSS2, and ADCK4) (6). Because genetic mapping data indicated a multitude of potential additional loci for SRNS, we here performed whole exome sequencing (WES) to identify additional monogenic SRNS genes and identified 9 different mutations in sphingosine-1-phosphate lyase (*SGPL1*) in 7 families as causing a previously unrecognized syndromic SRNS with a combination of ichthyosis/acanthosis, adrenal insufficiency, immunodeficiency, and/or neuronal dysfunction. We characterized mechanisms of molecular loss of function for the mutations detected and implicated sphingosine-1-phosphate (S1P) metabolism in the pathogenesis of SRNS. The syndromic features resulting from *SGPL1* mutations indicate the pivotal role of S1P metabolism in multiple tissues including kidney.

Results

SGPL1 mutations cause nephrotic syndrome, ichthyosis, facultative adrenal insufficiency, immunodeficiency, and neurologic defects in humans. Using WES in 7 families with a disease phenotype of SRNS, with adrenal insufficiency, ichthyosis-like acanthosis, immunodeficiency, or neurologic abnormalities (Table 1, Table 2, and Supplemental Figures 1–3; supplemental material available online with this article; https://doi.org/10.1172/JCI89626DS1), we identified recessive mutations in *SGPL1* (Figure 1, A–E). Homozygosity mapping (HM) in a Pakistani family (A280) (Supplemental Figure 1) with 3 siblings yielded a nonparametric lod score peak on chromosome 10, which did not coincide with any of the known recessive SRNS loci (Figure 1A) (7). Using WES, we detected a homozygous missense mutation (p.Arg222Gln) in a highly conserved (*Caenorhabditis elegans*) amino acid residue encoded by *SGPL1* (Table 1 and Figure 1, B–E). Notably, affected individuals from another consanguineous family (EB) showed a homozygous missense mutation involving the same amino acid residue (p. Arg222Trp), but presented with a more severe phenotype, with neonatal onset and profound immunodeficiency (Table 1 and Figure 1, B–E). A homozygous frameshift mutation of *SGPL1* (p.Ser3Lysfs*11) (Table 2 and Figure 1, B–E) was identified in affected individuals from family A5444, showing a phenotype of SRNS and cortisol deficiency. In family B46/B56, in whom 3 siblings had congenital nephrotic syndrome (NS) and severe extrarenal involvement combined with lymphopenia (Table 2 and Supplemental Figure 1), WES detected another homozygous missense mutation of *SGPL1* (p.Ser346Ile) (Table 2 and Figure 1, B–E) and in another individual with a related disease phenotype, B1245, a homozygous missense mutation, p.Tyr416Cys (Table 2 and Figure 1, B–E), with both mutations evolutionarily conserved to *S. cerevisiae*. In an individual MC with mononeuritis multiplex, SRNS, lymphopenia, and progressive contractures of extremities, WES identified compound heterozygous mutations in *SGPL1* (Table 2 and Figure 1, B–E). Both mutations are missense and affect highly conserved amino acid residues.

Patients from the nonconsanguineous family NCR61 (Table 1 and Figure 1, B–E) were found to be compound heterozygous for a frameshift mutation (p.Arg278Glyfs*17) and a missense mutation (p.Glu132Gly) not predicted to be deleterious by the PolyPhen (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) prediction tools, but predicted to induce skipping of exon 5 due to the introduction of additional exon splicing silencer elements (EX-SKIP, http://ex-skip.img.cas.cz) (8). Fibroblasts of patient NCR61-1 had almost no SGPL1 protein detectable by Western blot at the predicted size (Supplemental Figure 4A). Therefore, we hypothesized that the deleterious effect of c.395A>G was indeed related to the predicted RNA splicing defect and not to the p.Glu132Gly amino acid substitution. Amplification of the patient cDNA between exons 4 and 8 by PCR revealed a light band at the expected size and an intense smaller band with a size corresponding to loss of exon 5 (Supplemental Figure 4B). Sequencing of the PCR products confirmed the absence of exon 5 for the abundant smaller fragment and showed that the normal size fragment corresponded to the frameshift allele p.Arg278Glyfs*17. Skipping of exon 5 leads to a frameshift at amino acid position 88 and a stop codon 24 amino acids downstream (p. Ile88Thrfs*25).

By high-throughput exon sequencing (5, 9) in a worldwide cohort of approximately 800 additional families with NS, we did not detect any additional families with biallelic mutations of *SGPL1*. In all 7 families with *SGPL1* mutations (Tables 1 and 2 and Figure 1, B–K), direct inspection of sequence alignments did not yield a mutation in any of the 40 known SRNS genes. Most mutations identified in this study were absent from more than 60,000 control individuals in the ExAC server (http://exac.broadinstitute.org/) (Tables 1 and 2). Two missense mutations (c.665G>A; p.Arg222Gln and c.1247A>G; p.Tyr416Cys) are reported in the ExAC server, but their allele frequencies are extremely rare and they never occur in the homozygous state (Tables 1 and 2). All mutations segregated with the disease phenotype (Supplemental Figures 1 and 2). We thereby identified recessive *SGPL1* mutations as a cause of syndromic SRNS with ichthyosis/acanthocystosis, adrenal insufficiency, immunodeficiency, or neurologic involvement. We introduced the term NPHS14 for this syndromic SRNS caused by mutations of *SGPL1*.

In the majority of cases, NS, manifested as congenital NS or in the first year of life, showed no response to steroid therapy and

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Table 1. Five different mutations in *SGPL1* in 4 families with SRNS, ichtyosis, and facultative adrenal insufficiency, immunodeficiency, and neurologic defects

Family Individual	Nucleotide change	aa change	Exon (zygosity, segregation)	MT	aa conservation to species	ExAC allele frequencies	Sex	Ethnic origin	PC	Age of onset of SRNS (of ESRD)	Renal biopsy (at age)	Extrarenal manifestations
A5444	c.7dup	p.Ser3Lysfs*11	Exon 2 (HOM)	NA	NA	ND		Spanish Roma	Y			
07-306							M			4 yr	FSGS (4 yr)	Skin: ichthyosis (9 yr) Adrenal: no cortisol deficiency
MCM							M			Hematuria and proteinuria (7 mo) (transplant 5 yr); died at 8 yr	FSGS (17 mo)	Adrenal: bilateral adrenal calcifications (4 mo), AI (19 mo) Immuno: deficiency of cellular immunity (2 yr) Bones: craniotabes and rachitic rosary (5 mo) Neurol: sudden strabismus, abnormal gait (15 mo)
07-142							F			SRNS (10 mo) (first transplant, 5 yr; second, 12 yr]) relapse with massive protein-uria (15 yr)	FSGS (1 yr)	Skin: scaly lesions, hyperchromic skin (2 yr), ichthyosis vulgaris (5 yr), no skin biopsy Adrenal: glucocorticoid deficiency (11 yr) Neurol: cNMR normal (11 yr) Bones: acrosomia, asymmetric skull, moderate scoliosis
NCR61	c.395A>G	p.Glu132Gly Exon 5 skipping (p.Ile88Thrfs*25)	Exon 5 (het, p)	DC	*D. m.*	ND		France	N			
	c.832delA	p.Arg278Glyfs*17	Exon 10 (het, m)	NA	NA	ND						
1							F			2 yr (6 yr)	FSGS (2 yr)	Skin: ichthyosis Adrenal: AI (2 yr)
2							F			1 yr (4 yr)	FSGS (1 yr)	Skin: ichthyosis Adrenal: AI (2 yr)
A280	c.665G>A	p.Arg222Gln	Exon 8 (HOM)	DC	*C. e.*	2/120,744		Pakistan	Y			
21						(no homozygote)	M			19 yr	FSGS (19 yr)	Adrenal: AI (6.5 yr)
22							M			3 yr (7 yr), MHU (transplant 8 yr)	FSGS (3 yr)	Skin: calcinosis cutis Adrenal: AI (3 yr) Bones: SS Neurol: ptosis (25 yr), progressive defects, median and ulnar nerve paralysis
23							M			2 yr (2.9 yr), died at 2.9 yr	FSGS (2 yr)	Adrenal: AI (2 yr)
EB	c.664C>T	p.Arg222Trp	Exon 8 (HOM)	DC	*C. e.*	ND		Turkish	Y			
1							F			CNS (1 mo), died at 5 mo	ND	Adrenal: AI (3 mo) Immuno: immunodeficiency, lymphopenia (T and B cells) Neurol: microcephaly, hypoplasia of corpus callosum Other: anemia, failure to thrive, capillary leak syndrome
2							F			CNS, died at 2 mo	ND	Fetal hydrops
3							NA			Fetal demise	NA	ND
4							NA			Fetal demise	NA	ND

AI, adrenal insufficiency; *C. e., C. elegans;* DC, disease causing; *D. m., Drosophila melanogaster*; F, female; het, heterozygous; HOM, homozygous; immuno, immunological; MHU, microscopic hematuria; M, male; m, maternal; MT, mutationtaster; N, no; NA, not applicable; ND, no data; neurol, neurological; p, paternal; PC, parental consanguinity; SS, short stature; Y, yes.

Table 2. Four different mutations in *SGPL1* in 3 families with SRNS, ichtyosis, and facultative adrenal insufficiency, immunodeficiency, and neurologic defects

Family Individual	Nucleotide change	aa change	Exon (zygosity, segregation)	MT	aa conservation to species	ExAC allele frequencies	Sex	Ethnic origin	PC	Age of onset of SRNS (of ESRD)	Renal biopsy (at age)	Extrarenal manifestations
B56/B46	c.1037G>T	p.Ser346Ile	Exon 11 (HOM)	DC	*S. c.*	ND		Morocco	Y			
B46							F			CNS (3 mo), died at 6 mo	DMS (6 mo)	Prenatal edema/hydrops Skin: ichthyosis (at birth) Adrenal: small calcifications Neurol: microcephaly, muscular hypotonia, DD, deafness Other: hypothyroidism, intestinal malrotation, facial dysmorphism (microstomia; hypetelorism, epicanthus, dysplastic ears), hypocalcemia Immuno: lymphopenia
B56 (12M15411)							F			CNS, died at 1 mo	DMS (1 mo)	Skin: ichthyosis Neurol: microcephaly, deafness, muscular hypotonia, DD Other: fetal hydrops Immuno: lymphopenia
23							F			Fetal demise (36 wk GA)	NA	Hydrops
24							F			Fetal demise with hydrops fetalis	NA	Skin: ichthyosis
26							F			CNS (1 mo), died at 3 mo	NA	Adrenal: calcifications Neurol: microcephaly, seizures (3 mo) Immuno: lymphopenia Other: hypothyroidism, hypocalcemia
B1245	c.1247A>G	p.Tyr416Cys	Exon 12 (HOM)	DC	*S. c.*	1/121,408		Mixed European	Y			
21						(no homozygote)	F	(Hutterite)		CNS (2 mo) ESRD (5 mo) PD (2 mo)	FSGS (2 mo)	Adrenal: AI (2 mo) Neurol: bilateral sensorineural hearing loss (3 yr) Immuno: T and B cell lymphopenia, anemia (2 mo) Other: failure to thrive, DD, hypothyroidism (2 mo), mild dilated cardiomyopathy, Family history of 2 related children who died at young age of suspected immunodeficiency
MC	c.605C>T c.946G>A	p.Ser202Leu p.Ala316Thr	Exon 7 Exon 11 (comp het, inherited)	DC DC	*C. e.*[A] *C. e.*	ND ND	M	Mixed European (US)	N	18 yr (18 yr)	FSGS (18 yr)	Adrenal: none Neurol: peripheral neuropathy (motor and sensory), mononeuritis multiplex Immuno: T, B, NK lymphopenia; hypogammaglobulinemia; thrombocytopenia Other: elevated TSH, amblyopia, strabismus

[A]Except Xenop has Ala. AI, adrenal insufficiency; *C. e., C. elegans;* DC, disease causing; DD, developmental delay; F, female; het, heterozygous; HOM, homozygous; GA, gestational age; immuno, immunological; M, male; m, maternal; MT, mutationtaster; N, no; NA, not applicable; ND, no data; neurol, neurological; p, paternal; PC, parental consanguinity; PD, peritoneal dialysis; *S. c., Saccharomyes cerevisiae;* Y, yes.

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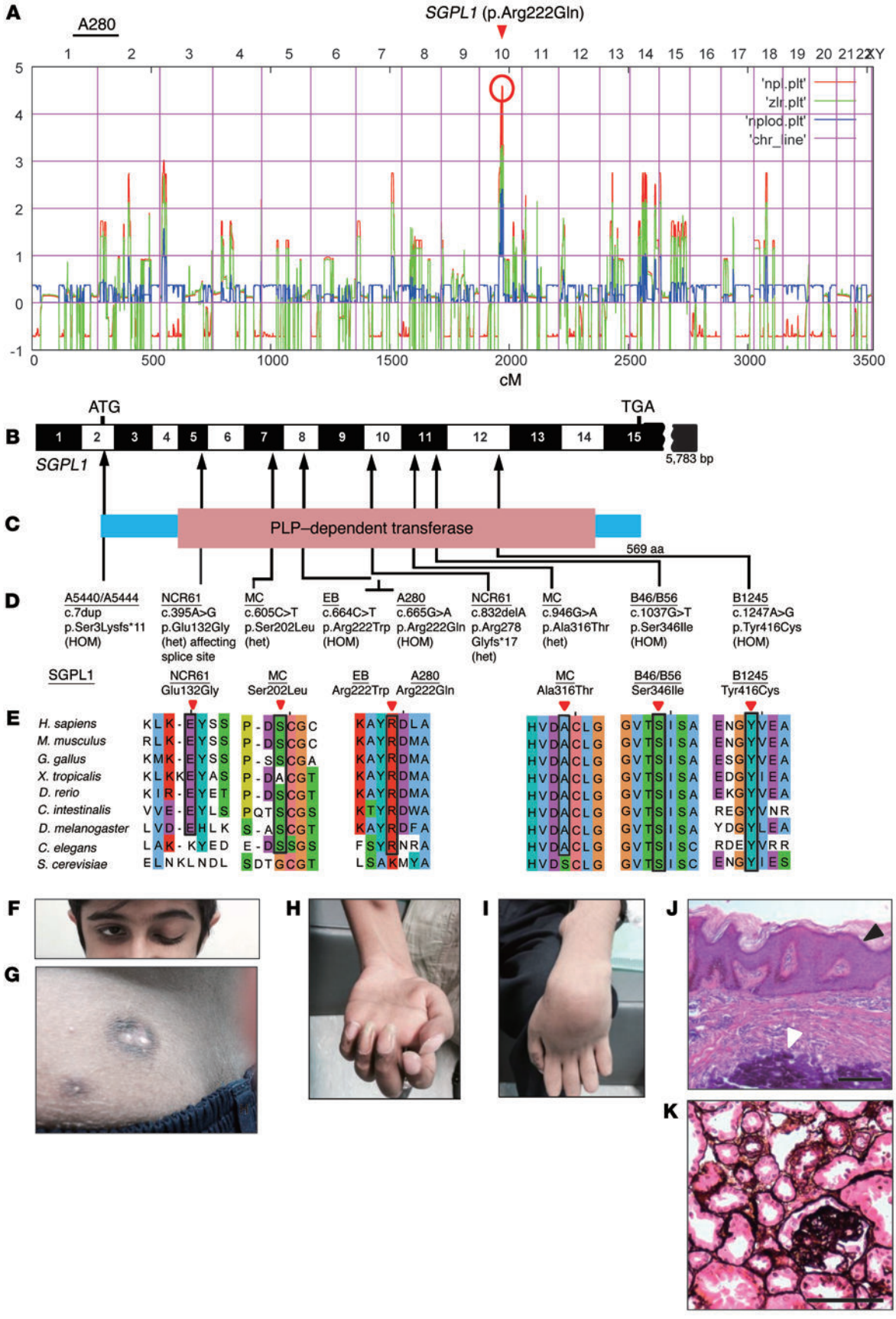

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Figure 1. HM and WES reveal *SGPL1* mutations as causing SRNS with ichthyosis and facultative adrenal insufficiency or neurologic defects (NPHS type 14). (**A**) Nonparametric lod scores across the human genome in 3 siblings of consanguineous family A280 with SRNS, acanthosis, and ichtyosis with facultative adrenal insufficiency. The *x* axis shows single-nucleotide polymorphism positions on human chromosomes concatenated from p-ter (left) to q-ter (right). Genetic distance is given in cM. The *SGPL1* locus (arrowhead) is positioned within the maximum nonparametric lod peak on chromosome 10. (**B**) Exon structure of human *SGPL1* cDNA. *SGPL1* contains 15 exons. Positions of start codon (ATG) and of stop codon (TGA) are indicated. (**C**) Domain structure of SGPL1. The extent of the PLP-dependent transferase domain is shown. (**D**) Five homozygous (HOM) and 4 compound-heterozygous *SGPL1* mutations (het) detected in 7 families with NPHS type 14. Family numbers (underlined), mutations, and predicted translational changes are indicated (see also Tables 1 and 2). (**E**) Evolutionary conservation of altered amino acid residues of SGPL1. Note that c.395A>G also resulted in p.Ile88Thrfs*25 through exon 5 skipping. (**F**) Ptosis in individual A280-22. (**G**) Skin image from individual A280-22 showing brownish black desquamation on sebostatic skin with multiple radial papules with a blueish/black erythema and central calcinosis. (**H** and **I**) Median (**H**) and ulnar nerve (**I**) paralysis in individual A280-22. (**J**) H&E-stained epidermal section from individual A280-22 showing acanthosis/orthokeratotic hyperkeratosis (black arrowhead) and calcinosis (white arrowhead). (**K**) Renal histology (silver staining) of individual A280-22, showing FSGS. Scale bars: 100 μm.

rapidly progressed to end-stage renal disease (ESRD). Histologically, FSGS was the main finding, but diffuse mesangial sclerosis (DMS) was found in cases with congenital NS (Tables 1 and 2 and Supplemental Figure 3). Extrarenal manifestations included ichthyosis and primary adrenal insufficiency, present in almost all cases except in those patients deceased at very early ages. About half of the affected individuals had a severe immunodeficiency. Patients presented with lymphopenia and multiple bacterial infections. A more detailed immunological work-up performed in patient EB-1 revealed severe lymphopenia with markedly reduced CD4 and CD8 T lymphocytes as well as B lymphocytes. In vitro cytokine generation upon stimulation was also deficient (Supplemental Table 3). Neurological deficits were also present in half of the patients and included sensorineural deafness, microcephaly (Supplemental Figure 3), corpus callosum hypoplasia in 1 case, peripheral nerve paralysis of the median and ulnar nerves, and ptosis of the left eye (Tables 1 and 2 and Figure 1, F–I). In a few cases, dysmorphic features and bone defects were also present. Two families had a history of fetal demise and hydrops fetalis.

SGPL1 encodes S1P lyase, an intracellular enzyme responsible for the final step in sphingolipid breakdown, converting its main substrate S1P into ethanolamine phosphate and hexadecenal. S1P is a bioactive sphingolipid that acts extracellularly by binding to protein-coupled receptors of the lysophospholipid receptor family and intracellularly through S1P receptor–independent (S1PR-independent) mechanisms. Through receptor activation, S1P mediates autocrine and paracrine signals, controlling cell migration and proliferation as well as lamellipodia dynamics (10). An *Sgpl1*-deficient mouse model exhibits glomerular proteinuria, a skin phenotype of acanthosis with orthokeratotic hyperkeratosis and platelet activation (11).

Sgpl1 localizes to the endoplasmic reticulum of renal glomerular cells. All of the 40 monogenic genes that, when mutated, cause SRNS are highly expressed in podocytes. We therefore examined cell-type–specific and subcellular localization of SGPL1 in mouse kidney. To assess the specificity of an anti-SGPL1 antibody used in immunofluorescence, we first stained kidney sections of $Sgpl1^{-/-}$ and $Sgpl1^{+/+}$ mice (Figure 2, A and B). Most of the SGPL1 signal observed in kidneys of $Sgpl1^{+/+}$ mice was absent from kidneys of $Sgpl1^{-/-}$ mice (Figure 2A), demonstrating specificity of the signal. To determine cellular localization of SGPL1, immunofluorescence was performed using various cell- and organelle-specific markers (Figure 2, C–F). SGPL1 localized to podocytes, whose nuclei were marked with WT1 (Figure 2C). As shown by the colocalization of SGPL1 with the ER marker BiP, it localized to ER in podocytes, although this colocalization was more obvious in proximal tubules (Figure 2C). In addition, SGPL1 appeared to be present in other renal glomerular cell types, for example, in mesangial cells stained with α–smooth muscle actin (Figure 2E) and endothelial cells stained with CD31 (Figure 2F).

In silico modeling of SGPL1 missense mutations. To predict potential structural changes in SGPL1 protein that might arise from the mutations (p.Arg222Gln and p.Ser346Ile) detected in patients with NPHS type 14 Tables 1 and 2), we performed in silico analyses (Figure 2, G and H). SGPL1 forms a symmetric homodimer. Two subunits form a tightly intertwined dimer with both chains contributing to the catalytic cavity defined by the covalently bound cofactor pyridoxal phosphate (PLP) (Figure 2, G and H). Arg222 is located at the symmetric dimer interface and forms 2 important hydrogen bonds with the carbonyl backbone moieties of Tyr250 and Ser249, contributing to the binding affinity of the homodimer. The p.Arg222Gln mutation leads to a loss of these 2 hydrogen bonds in both chains (Figure 2G), which is reflected in the delta affinity being predicted as unfavorable by about 10 kcal/mol. In addition, the mutation seems to be destabilizing the protein, since Arg222 also forms hydrogen bonds with adjacent residues in its own symmetric dimer chain. Ser346 is not at the dimer interface, but buried within each chain (Figure 2H). Its hydroxyl group is involved in 2 hydrogen bonds, accepting one from Tyr221 and donating one onto His313 (Figure 2H). The p.Ser346Ile mutation is predicted to be considerably disfavored because it not only breaks this hydrogen-bond network, but because isoleucine is also much bulkier than serine, leading to steric clashes with its surrounding residues (Figure 2H). Hence the delta stability is predicted to be +130 kcal/mol.

Mutations alter subcellular localization of SGPL1. Because in silico analysis predicted that the p.Arg222Gln mutation may affect the interface of the SGPL1 homodimer, we examined this hypothesis by coimmunoprecipitation. We transfected HEK293T cells with plasmids with 2 alternative tags (Myc and FLAG). Compared with WT SGPL1, p.Arg222Gln and p.Ser346Ile mutant proteins exhibited reduced expression levels, whereas p.Glu132Gly had a similar expression level upon overexpression (Figure 2I and Supplemental Figure 5). Neither missense (p.Glu132Gly, p.Arg222Gln, and p.Ser346Ile) nor truncating (p.Arg278Glyfs*17) mutations abrogated dimer formation (Figure 2I and Supplemental Figure 5). However, when compared with overexpression of the SGPL1 WT construct, overexpression of p.Arg222Gln, p.Ser346Ile, and p.Arg278Glyfs*17 SGPL1 proteins in HEK293T cells led to abnormal cytoplasmic aggregates (Figure 2J). Aggregation of SGPL1 mutant proteins was confirmed upon overexpression in human podocytes (Supplemental Figure 6).

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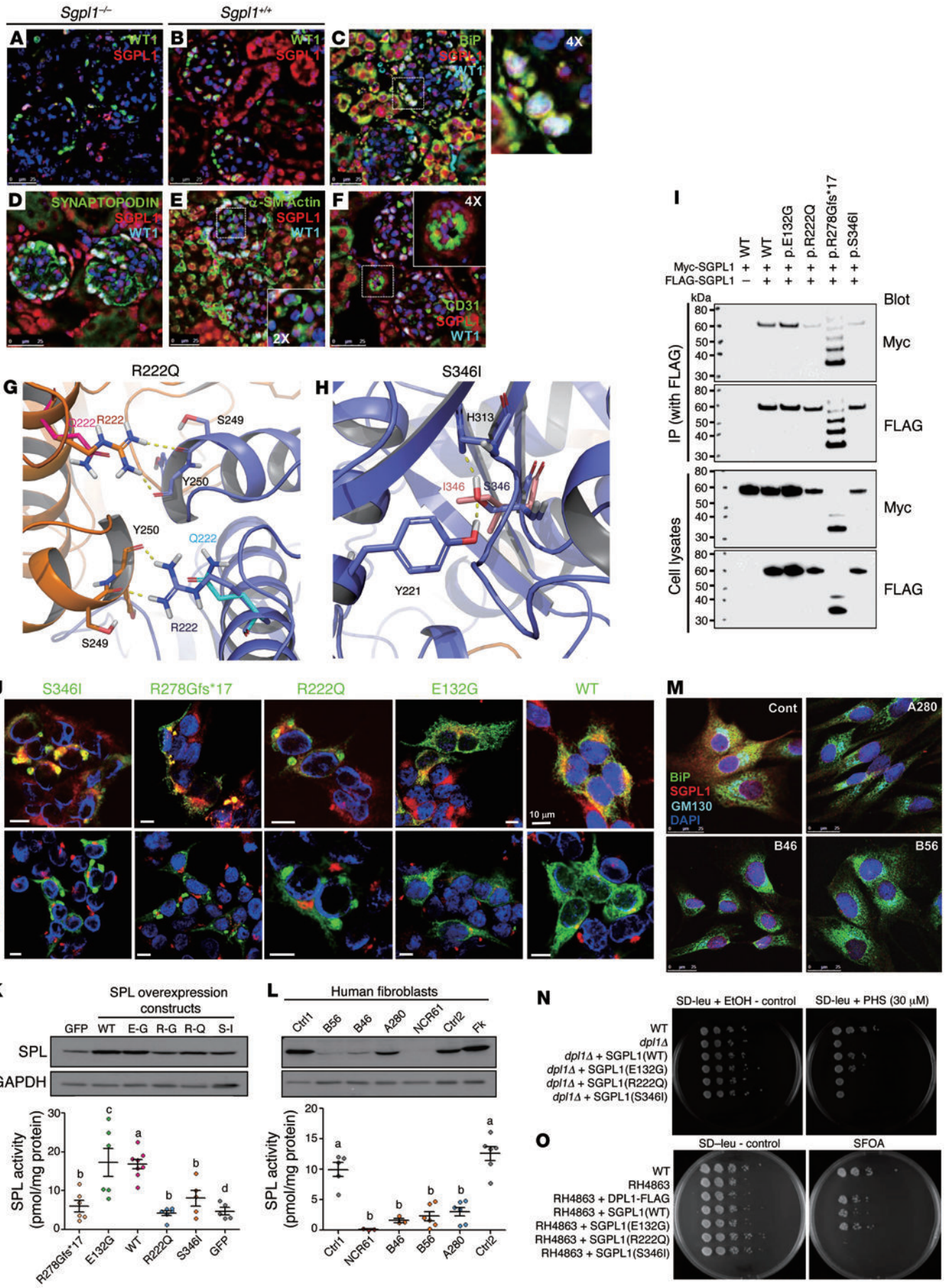

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Figure 2. Biological and biochemical consequences of recessive *SGPL1* mutations. (**A**–**F**) Kidney sections of *Sgpl1*$^{-/-}$ mice (**A**) and *Sgpl1*$^{+/+}$ mice (**B**) were stained with anti-SGPL1 (red) and WT1 antibodies (green). (**B**–**F**) Coimmunofluorescence of SGPL1 with marker proteins (green) in *Sgpl1*$^{+/+}$ kidney: podocyte (**B**, WT1), ER (**C**, BiP), podocyte foot processes (**D**, synaptopodin), mesangial cells (**E**, α–smooth muscle actin), and endothelial cells (**F**, CD31). Scale bars: 25 μm. (**G** and **H**) Structural modeling of *SGPL1* mutations. The 2 monomers of the SGPL1 homodimer are shown in the drawing in blue and orange, respectively. (**G**) p.Arg222Gln; (**H**) p.Ser346Ile. (**I**) Coimmunoprecipitation to assess dimerization of WT vs. mutant SGPL1 proteins (see also Supplemental Figure 5). Coimmunoprecipitation is representative of 3 experiments. (**J**) Mislocalization of variant SGPL1 proteins upon overexpression in HEK293T cells. BiP (red), or Golgi marker GOLGB1 (red), and anti-Myc antibody (green). Scale bars: 10 μm. (**K**) SGPL1 enzyme activity levels in transformed HEK293T cells. HEK293T cells expressing a GFP indicate endogenous SGPL1 activity levels (a vs. b and a vs. d, $P < 0.0025$; c vs. d, $P = 0.013$; a vs. c, no significant difference). (**L**) SGPL1 protein expression and enzyme activity levels in fibroblasts from 2 control individuals (Ctrl 1 and 2), normal human foreskin fibroblasts (Fk), and 4 individuals with *SGPL1* mutations. (a vs. b, $P < 0.0001$.) Results are from the averages of triplicates in **K** and **L**. (**M**) Immunofluorescence of SGPL1 in fibroblasts. BiP (green), Golgi marker GM130 (blue), and anti-SGPL1 antibody (red). Scale bars: 25 μm. (**N**) PHS toxicity test. Ability to complement *dpl1Δ* deletion on medium containing PHS was tested for human SGPL1 WT and mutants. (**O**) Synthetic lethality test. Human WT and p.Glu132Gly SGPL1 expressing RH4863 survived on 5-FOA plates. However, p.Arg222Gln and p.Ser346Ile mutants did not allow for survival of *DPL1* (*SGPL1*) deficient strains.

Mutations in SGPL1 result in decreased expression and reduced lyase activity. To assess whether mutations detected in individuals with NPHS type 14 altered SGPL1 enzyme activity, we measured SGPL1 and enzyme activity levels in HEK293T cells transiently expressing either WT or mutant SGPL1 (Figure 2K). We found that overexpression of the p.Arg222Gln, p.Ser346Ile, and p.Arg278Glyfs*17 mutant alleles resulted in strongly reduced enzyme activity levels, whereas the p.Glu132Gly mutant (c.395A>G) did not, consistent with our finding that c.395A>G leads to skipping of exon 5 and protein truncation (p.Ile88Thrfs*25) (Supplemental Figure 4). We also found that protein expression levels and corresponding SGPL1 enzyme activity levels in patient dermal fibroblasts (Figure 2L) exhibited reduced SGPL1 enzyme activity for individuals B46 (p.Ser346Ile), B56 (p.Ser346Ile), NCR 61-1 (p.R278fs*17 and p.Ile88Thrfs*25), and A280-21 (p.Arg222Gln) compared with controls (Figure 2L). In addition, there was reduced expression of SGPL1 protein when examined by immunofluorescence microscopy in patient fibroblasts (Figure 2M). Taken together, these results suggest that the primary impact of the patient's mutations is on SGPL1 expression level and protein stability.

SGPL1 mutations fail to rescue growth in DPL1-deficient yeast. Functionality of human WT and mutant SGPL1 proteins were tested in an in vivo yeast complementation assay by measuring their ability to complement the deletion of the *SGPL1* yeast ortholog *DPL1* (*dpl1Δ*) on medium containing phytosphingosine (PHS) (Figure 2N and Supplemental Figure 7A). Inability to degrade toxic long-chain bases led to decreased viability and slowed growth. Human WT and p.Glu132Gly mutant SGPL1 were found to be able to partially restore *dpl1Δ*, while p.Arg222Gln, p.Ser346Ile, and p.Tyr416Cys as well as frameshift mutants p.Arg278Glyfs*17 and p.Ser3Lysfs*11 showed no improved growth compared with *dpl1Δ* (Figure 2N and Supplemental Figure 7A), consistent with our finding that the c.395A>G allele most likely conveys loss of function through its splice defect (Supplemental Figure 4B). The results were also reproduced on plates containing C17 and C18 dihydrosphingosine (DHS).

To further investigate functional consequences of *SGPL1* mutants, we carried out a synthetic lethality assay in yeast (Figure 2O and Supplemental Figure 7B) using an RH4863 strain in which *DPL1* and *LCB3* were deleted, rendering the strain synthetically lethal. Viability of the strain was maintained by expressing *LCB3* from a *URA3* plasmid. We tested functionality of *SGPL1* and corresponding mutants expressed in RH4863 by forcing them to lose *LCB3*-coding *URA3* plasmid on plates containing 5-fluoroorotic acid (5-FOA), which selects against *URA3*-expressing cells. Survival on 5-FOA plates indicates that cells have regained the ability to degrade long-chain bases and thus compensate for the loss of *LCB3*. In agreement with the PHS toxicity test, synthetic lethality assay demonstrated functional integrity of WT and p.Glu132Gly mutant SGPL1 proteins, but not p.Arg222Gln, p.Ser346Ile, p.Tyr416Cys, p.Arg278Glyfs*17, and p.Ser3Lysfs*11 (Figure 2O and Supplemental Figure 7B).

SGPL1 mutations alter ceramide composition of patient fibroblast-conditioned medium. S1P participates in regulating multiple cellular processes, and its intracellular levels are tightly regulated. Since SGPL1 controls the only exit point for sphingolipid catabolism, inactivation of the enzyme can result in accumulation of various bioactive sphingolipid intermediates, including phosphorylated and nonphosphorylated sphingoid bases and ceramides.

Conditioned medium from patient fibroblast cultures showed significantly elevated C22:0, C24:0, and C24:1 ceramides compared with control fibroblast-conditioned medium, whereas S1P levels were below the limits of detection (Supplemental Figure 8). These very long chain ceramides are produced by ceramide synthase 2 (12).

Sgpl1$^{-/-}$ *mice exhibit podocyte foot process effacement*. *Sgpl1*$^{-/-}$ mice exhibit glomerular proteinuria, a skin phenotype of acanthosis with orthokeratotic hyperkeratosis, platelet activation, and immunodeficiency (11). In order to determine the renal histology of *Sgpl1*$^{-/-}$ mice, we performed transmission electron microscopy (TEM) of kidneys harvested from *Sgpl1*$^{+/+}$ and *Sgpl1*$^{-/-}$ mice to directly examine glomerular structures upon loss of *Sgpl1*. *Sgpl1*$^{-/-}$ kidneys exhibited complete foot process effacement and absence of slit diaphragms (Supplemental Figure 9). In addition, *Sgpl1*$^{-/-}$ mice exhibited hypoalbuminemia (serum albumin KO 2.2 ± 0.6 g/dl vs. WT or heterozygote [HET] 3.7 ± 0.4 g/dl, $n = 4$ KO and $n = 6$ WT or HET) and an elevated urinary albumin/creatinine ratio (ACR) (KO 1176.4 ± 932.8 vs. WT or HET 103.5 ± 94.2, $n = 3$ KO, $n = 5$ WT or HET, P value, no significant difference).

However, when we examined apoptosis in response to S1P in cultured podocytes, we did not observe an effect in a dose range expected to be present in SGPL1-induced pathology (Supplemental Figure 10). Likewise, we did not observe any effect of *SGPL1* knockdown on podocyte migration rate, a pathogenic effect that has previously been demonstrated in many monogenic forms of SRNS (Supplemental Figure 11) (13).

Decreased mesangial cell migration rate upon SGPL1 knockdown is reversed by an S1PR inhibitor. Because we did not observe any cellular pathologic effect in cultured podocytes regarding apop-

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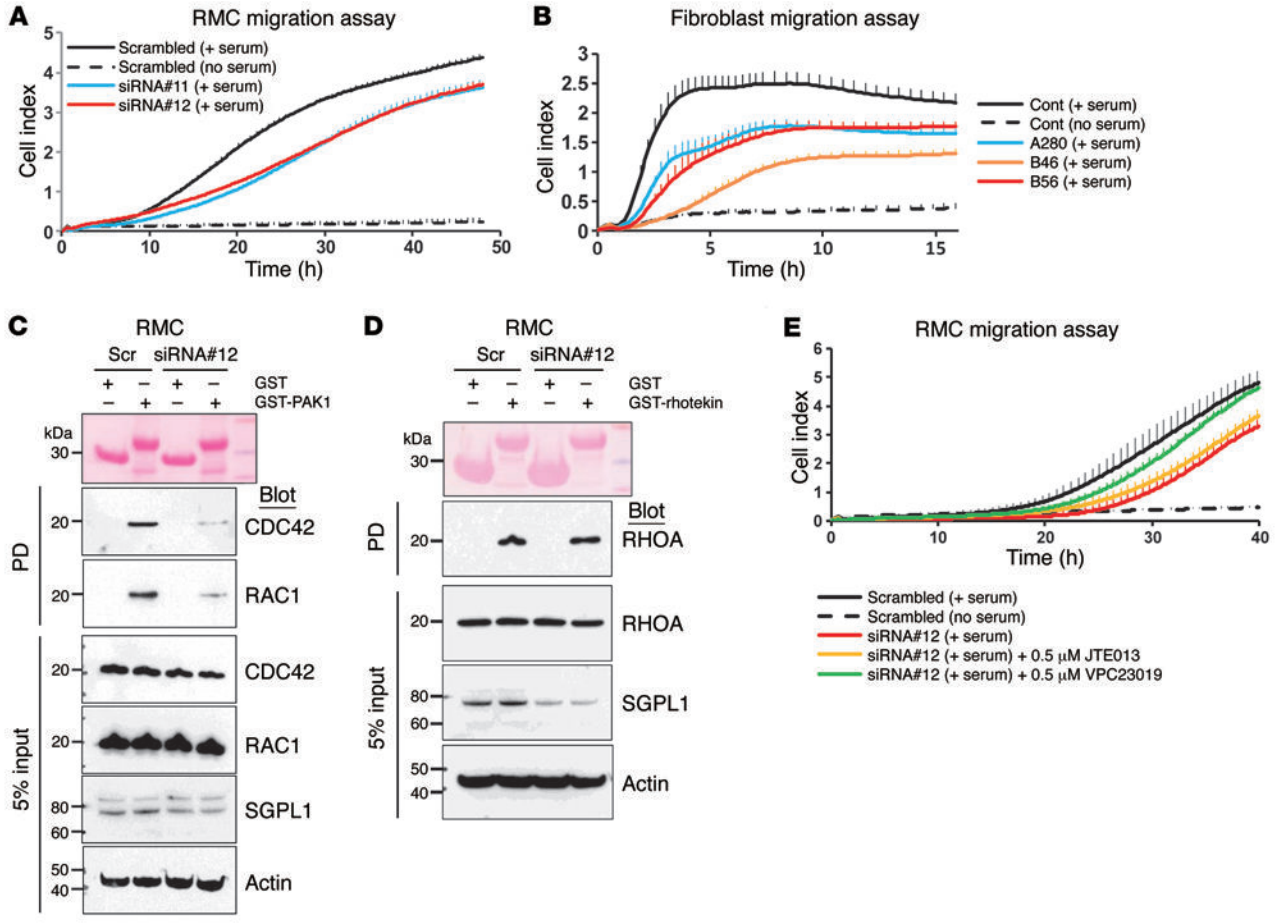

Figure 3. Effect of *Sgpl1* knockdown on RHO GTPase activity in RMCs and patient fibroblasts. (**A**) Effect of SGPL1 knockdown on rat RMCs using the xCELLigence system. RMC transfected with 2 different SGPL1 siRNAs exhibited decreased serum-induced migration rate (blue and red lines) compared with scrambled siRNA control (black line). (**B**) Diminished cell migration rate in patients with *SGPL1* mutations. Using the xCELLigence system, fibroblasts from individuals with *SGPL1* mutations (blue, red, and orange lines) showed decreased migration rate compared with control (black solid line). (**C**) Active GTP-bound RAC1 and CDC42 precipitated from RMCs transfected with scrambled (Scr) or *SGPL1* siRNA using a GST-PAK1 (CRIB) pulldown assay. Compared with control cells, RMCs transfected with SGPL1 siRNA exhibited a significant decrease in relative CDC42 and RAC1 activity. The efficiency of knockdown by siRNA was confirmed by immunoblotting with an anti-SGPL1 antibody (second to lowest panel). (**D**) Active GTP-bound RHOA precipitated from RMCs using a GST-rhotekin (RBD) pulldown assay. RMCs transfected with scrambled control siRNA versus *SGPL1* siRNA exhibited no significant differences in relative RHOA activity. **C** and **D** represent 3 experiments each. (**E**) Effect of S1PR antagonists on RMC migration rate. SGPL1 knockdown caused decreased migration rate (red line) (Supplemental Figure 3B), which was partially rescued by VPC23109 (green line), but not by JTE013 (orange line). VPC23109 is an antagonist that selectively inhibits S1PR1 and S1PR3, whereas JTE013 is an antagonist for S1PR2. Each cell index value corresponds to the average of more than triplicates and SD is in only 1 direction for clarity in **A**, **B**, and **E**.

tosis or cell migration, because SGPL1 was present in mesangial cells (Figure 2E), and because *Sgpl1*$^{-/-}$ mice exhibited reduced glomerular mesangial cell numbers (11), we examined pathogenic effects of siRNA knockdown of *Sgpl1* in mesangial cells (Figure 3).

To test whether *SGPL1* or S1P is involved in cell survival or proliferation, we investigated the effect of silencing SGPL1 on cell apoptosis and proliferation in rat mesangial cells (RMCs). Knockdown of *Sgpl1* did not affect apoptosis or proliferation in RMCs (Supplemental Figure 12). We then examined the effect of *Sgpl1* knockdown on migration of RMCs and found that migration of RMCs was reduced (Figure 3A) upon knockdown of *Sgpl1* by transfection with either of 2 siRNAs (Supplemental Figure 12). In addition, we confirmed this effect by showing that cell migration was significantly reduced in fibroblasts from individuals with *SGPL1* mutations (Figure 3B).

S1P has previously been implicated in the regulation of RHO-like small GTPases (RHOA/RAC1/CDC42), and disruption of RAC1 signaling was previously implicated in the pathogenesis of SRNS (13–15). We therefore tested whether knockdown of *SGPL1* would affect activation of the RHO-like small GTPases. When *Sgpl1* was silenced by siRNA in RMCs, we observed a decrease in active GTP forms of CDC42 and RAC1 (Figure 3C), whereas active RHOA (Figure 3D) was unchanged.

To test for specificity of the migration defect observed upon *Sgpl1* knockdown, we employed S1PR antagonists. Among 5 S1PR (S1PR1-5), S1PR1, S1PR2, and S1PR3 are expressed in kidney (16). Therefore, we chose VPC23109, which preferentially acts on S1PR1 and S1PR3, and JTE013, which is an antagonist for S1PR2. Interestingly, VPC23109 partially rescued the decreased migration conferred by *Sgpl1* knockdown in RMCs, whereas JTE013 failed to

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rescue the migration defect, suggesting that the S1P effect on RMC migration rate is mediated through S1PR1 and/or S1PR3 (Figure 3E).

SGPL1 is required for Drosophila nephrocyte function. *Sply* is the *SGPL1* ortholog of *Drosophila*, and a *Sply* null allele has previously been characterized and shown to decrease fly viability (17). Accordingly, *Sply* null hemizygous flies (*Sply* null/*Df*) had decreased viability (Figure 4A).

To assess whether *Sply* deficiency evoked a podocytopathy phenotype in flies, we assessed the structure and function of nephrocytes in *Sply* null hemizygous flies. Nephrocytes are thought to represent the *Drosophila* counterparts of podocytes. These cells display invaginations of plasma membrane that form analogous structures to podocyte foot processes and are connected by a slit diaphragm (18). This structure allows filtering of hemolymph and macromolecules that can then undergo endocytosis. We observed no major decrease of the slit diaphragm protein kirre (NEPH1 ortholog) by immunofluorescence (Supplemental Figure 13); however, by TEM, we identified a reduction of foot process density (Figure 4C). To assess the impact of *Sply* on nephrocyte function, we analyzed the uptake of fluorescent-coupled albumin. Albumin is filtered and endocytosed in nephrocytes under normal conditions, but not when there is disruption of foot processes (18, 19). Consistently, *Sply* null hemizygous flies had significantly reduced uptake of albumin as compared with control (Figure 5A).

Due to the known role of *Sply* on sphingolipid catabolic pathway and its indirect implication in other lipid pathways, such as the regulation of sterol regulatory element-binding protein processing in *Drosophila* (20), we also studied the effect of *Sply* KO on the lipid content of nephrocytes. *Sply* null hemizygous flies showed an almost complete absence of lipid droplets stained by Bodipy (Figure 5B) or Nile red (data not shown), suggesting a reduction of neutral lipids. Using liquid chromatography–mass spectrometry (LC/MS), we also assessed the accumulation of sphingoid bases and ceramides in *Sply* null hemizygous flies. *Drosophila* has been shown to have C14 and C16 sphingosine (21) and C14 and C16 sphingadienes (22), a sphingoid base with an extra double bond compared with sphingosine. Sphingolipid intermediates upstream of S1P, including long chain bases and ceramides, have been shown to accumulate when SGPL1 activity is inhibited, including in *Sply* mutant flies (17, 22). As expected, we observed a significant increase of C16 sphingosine, C14 sphingadiene, and C16 ceramide in *Sply* null hemizygous third instar larvae (Figure 5C and Supplemental Figure 14). Together, these data suggest that *Sply* null hemizygous flies have defects reminiscent of the podocyte disease found in humans with *SGPL1* mutations and display altered lipid metabolism due to the disruption of sphingolipid catabolic pathway.

Lack of rescue with mutant alleles in the Drosophila model. To assess the effect of *SGPL1* missense mutations in vivo, we reexpressed the HA-tagged *Sply* carrying WT or the corresponding human mutations p.Glu119Gly, p.Arg210Gln, and p.Ser335Ile (equivalent to human mutants p.Glu132Gly, p.Arg222Gln, and p.Ser346Ile, respectively). We observed that under its endogenous promoter, HA-tagged *Sply* is expressed in nephrocytes (Figure 4B). Consistent with our previous results, the expression of mutants p.Arg210Gln and p.Ser335Ile was greatly reduced at the protein level compared with WT (Figure 4B), but without any significant difference at the mRNA level (Supplemental Figure 15).

Reexpression of *Sply* WT and p.Glu119Gly transgenes rescued *Sply* mutant viability defect and nephrocyte dysfunction, which was not the case for p.Arg210Gln and p.Ser335Ile mutations (Figure 4 and Figure 5A). Regarding the neutral lipid content of nephrocytes, we observed a rescue of the *Sply* phenotype with *Sply* WT and p.Glu119Gly transgenes, but not with p.Arg210Gln or p.Ser335Ile (Figure 5B).

In whole third instar larvae, the phenotype of C16 sphingosine and C16 ceramide accumulation present in *Sply* null hemizygous flies was rescued with equal efficiency by *Sply* WT and p.Glu119G transgenes (Figure 5C). However, p.Ser335Ile mutant failed to rescue (Figure 5C). p.Arg210Gln mutant partially rescued the *Sply* null phenotype, but with significantly less efficiency than *Sply* WT (Figure 5C). This suggests that p.Arg210Gln and p.Ser335Ile, but not p.Glu119Gly, mutations affect the *Sply* enzymatic activity and result in the accumulation of upstream sphingolipids, sphingosines, and ceramides. Similar results were observed when analyzing fly sphingadienes, although complementation results for the mutants p.Arg210Gln and p.Ser335Ile did not reach a statistically significant difference from control (Supplemental Figure 14). We conclude that SGPL1 missense mutations p.Arg222Gln and p.Ser346Ile are pathogenic, impair sphingolipid metabolism, and induce nephrocyte defects reminiscent of those found in human podocytopathies. Consistent with our data on the splicing defects (Supplemental Figure 4B) induced by p.Glu132Gly mutation, we found that the corresponding amino acid change did not induce any defect in *Drosophila*.

Discussion

In this study, we identified recessive *SGPL1* mutations as a cause of syndromic SRNS. The disease phenotype entailed SRNS with facultative ichthyosis, adrenal insufficiency, neurologic involvement, and immunodeficiency. We show that *SGPL1* mutations are loss-of-function mutations that lead to reduced protein levels and enzyme activity and impaired degradation of long-chain sphingoid bases. In *Drosophila*, the missense mutations led to decreased viability and nephrocyte defects that are reminiscent of podocyte changes in human NS. WT *Sply* complemented *Sply* phenotype and sphingolipid profiles, whereas *Sply* harboring mutations did not complement.

SGPL1 is ubiquitously expressed in tissues and is an essential enzyme of the sphingolipid catabolic pathway. It is thus considered a main regulator of S1P levels and other sphingoid bases. Loss of *Sgpl1* in mouse models phenocopies the human disease. In fact, *Sgpl1*$^{-/-}$ mice fail to thrive and die soon after weaning, showing defects in the immune system, the urinary system, vasculature, and bone as well as altered lipid metabolism (11, 23–25). However, adrenal insufficiency has not been described in *Sgpl1*$^{-/-}$ mice, but to our knowledge, adrenal function was not assessed in these animals. In the immune system, changes in local S1P concentration and gradient between tissues (low S1P) and efferent lymph and blood (high S1P) affect T cell egress from lymphoid organs (26, 27), resulting in reduced levels of circulating lymphocytes with overrepresentation of T cells with a memory phenotype over naive T cells (27), as observed in the SGPL1 mutant patient in which a complete immunological work-up has been performed. Interestingly, partial deficiency of *Sgpl1* in inducible knockout models led

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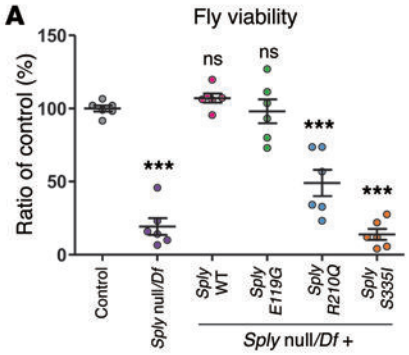

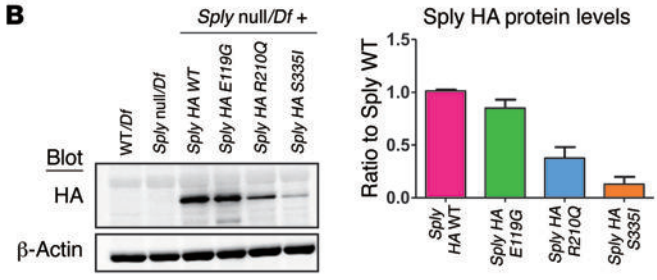

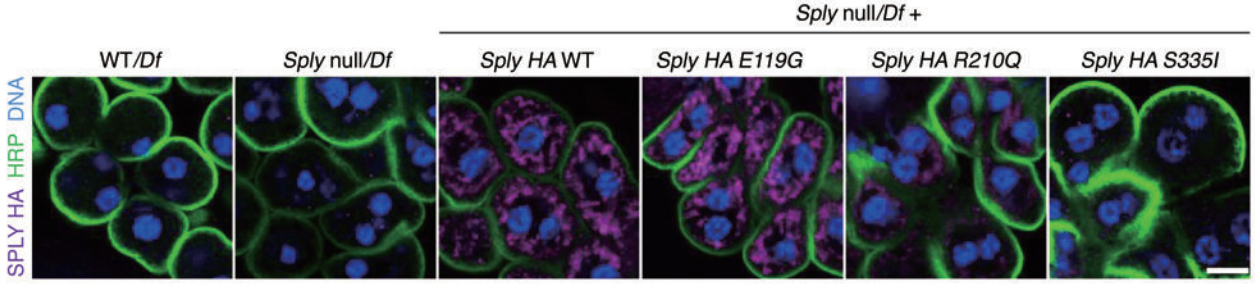

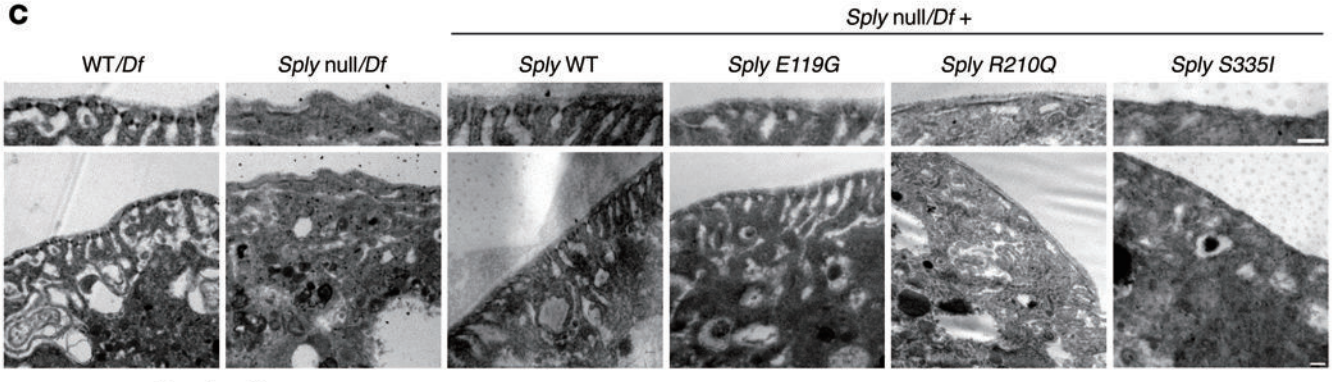

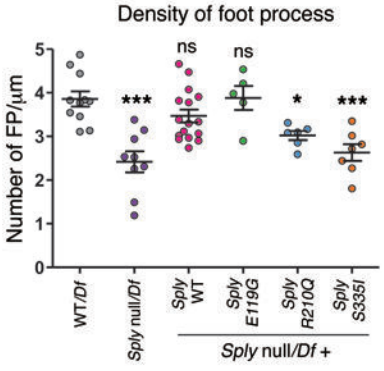

Figure 4. *SGPL1* missense mutations fail to rescue the phenotype of the Drosophila *SGPL1* ortholog (*Sply*) KO. Human *SGPL1* mutations p.Glu132Gly, p.Arg222Gln, and p.Ser346Ile are equivalent to *Drosophila Sply* mutations p.Glu119Gly, p.Arg210Gln, and p.Ser335Ile. (**A**) Viability defects of *Sply* null hemizygous and *Sply* mutant flies. Viability was calculated as the percentage of *Sply* null hemizygous offspring of heterozygous parents. Values are normalized to the viable control *Df(2R)BSC433/Df(2R)247*. More than 650 flies per genotype; 6 independent experiments. (**B**) Western blot of HA-tagged *Sply* in third instar larvae (top panel) and immunofluorescence of third instar garland nephrocytes stained for HA (purple) (bottom panel). Membrane and nuclei were labeled with HRP (green) and Hoechst (blue), respectively. Five or more larvae/genotype; 3 independent experiments. Scale bar: 10 µm. (**C**) Foot process density in *Sply* null hemizygous and *Sply* mutant third instar garland nephrocytes. TEM images and quantification. Six or more nephrocytes/genotype; 2 independent experiments. Scale bars: 200 nm. Statistical analysis performed by Bonferroni's test following ANOVA. ***$P < 0.0005$; *$P < 0.05$. All graphs show mean ± SEM. ns, not significant.

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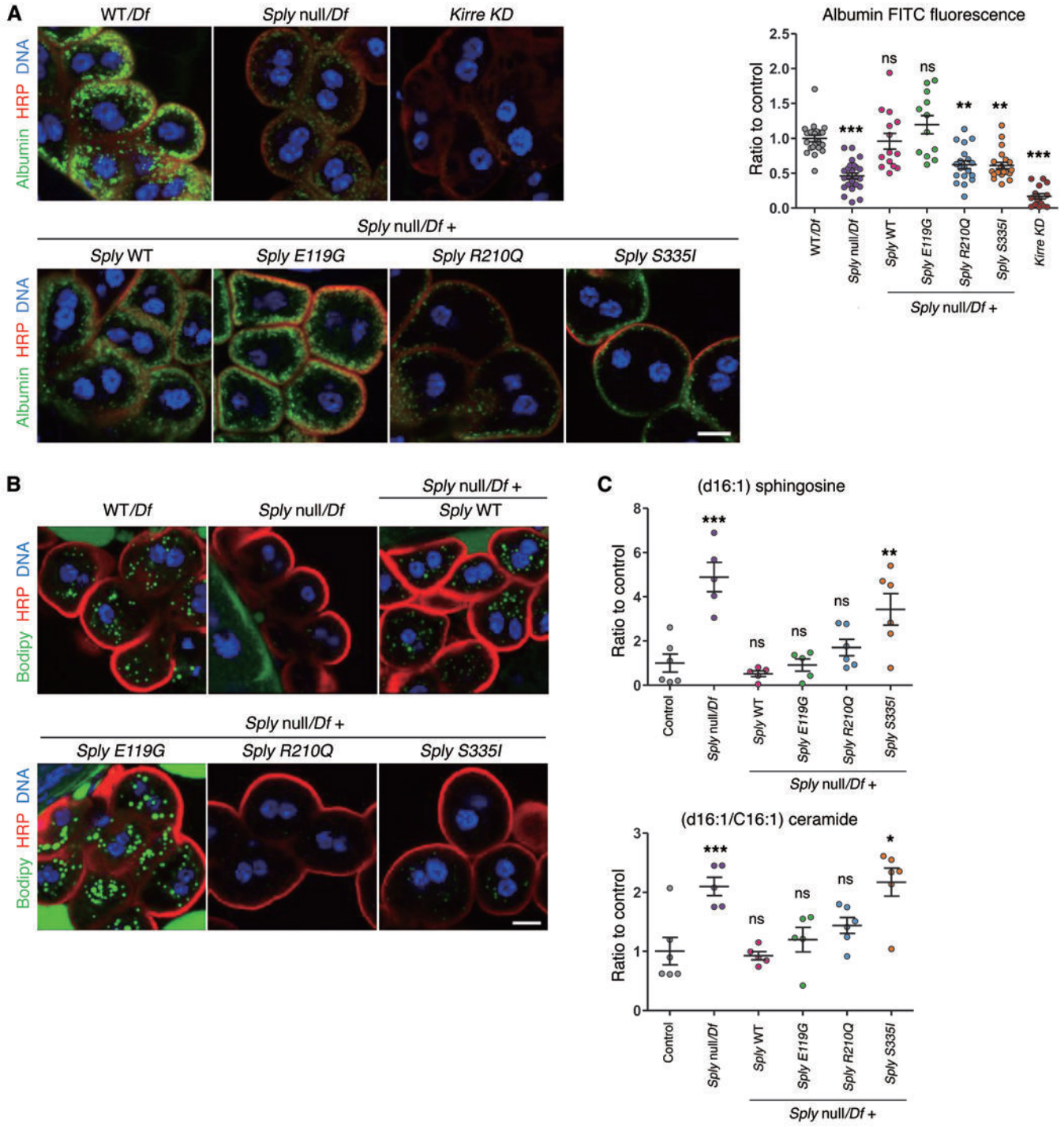

Figure 5. Defects of vesicular transport and sphingosine metabolism in the SGPL1 drosophila ortholog (*Sply*) KO. (**A**) Albumin uptake in *Sply* null hemizygous and *Sply* mutant third instar garland nephrocytes. Nephrocytes were incubated for 2.5 minutes with albumin-FITC (green), fixed and stained for the membrane marker HRP (red). Ten or more larvae/genotype; 3 independent experiments. Scale bar: 10 μm. (**B**) Lipid droplets in *Sply* null hemizygous and *Sply* mutant third instar garland nephrocytes, assessed by Bodipy staining. Six or more larvae/genotype; 2 independent experiments. Scale bar: 10 μm. (**C**) Sphingoid bases accumulation in *Sply* null hemizygous and *Sply* mutant third instar larvae assessed by LC/MS. Note that p.Arg210Gln mutant rescues with less efficiency than *Sply* WT (p.Arg210Gln vs. *Sply* WT, $P = 0.009$ for sphingosines and $P = 0.02$ for ceramides, *t* test). $n = 6$ independent experiments. For *Sply* null, *Sply* WT, and p.E119G, 1 analysis was removed due to poor quality chromatography. Control corresponds to WT larvae. Statistical analysis performed by Dunnet's (**C**) post-hoc tests following ANOVA or Dunn's post-hoc test following Kruskal-Wallis (**A**). $***P < 0.0005$; $**P < 0.005$; $*P < 0.05$. All graphs show mean ± SEM.

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to a less severe phenotype, with decreased lethality and in which prevailing manifestations involved kidney (massive proteinuria with FSGS), the immune system, platelets, and skin (28, 29). The variable organ involvement found in different *SGPL1* mutated families may be related to levels of residual functional SGPL1 conveyed by different mutations. However, the intrafamilial variability may be explained by the presence of other genetic factors that may influence the disease manifestations. For instance, variants or polymorphisms in genes involved in S1P metabolism and signaling, such as *SGPP1*, *SGPP2*, *SPHK1*, *SPHK2*, *SPNS2,* or *S1PR1-5*, may contribute to intrafamilial variability.

The phenotypic spectrum of *SGPL1* mutations is reminiscent of Schimke immunoosseous dysplasia (MIM 242900), which is caused by recessive mutations in *SMARCAL1* encoding SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like protein 1. Specifically, mutations in both *SMARCAL1* and *SGPL1* are associated with FSGS and T cell–related immunodeficiency. Whereas *SMARCAL1* is involved in reannealing stably unwound DNA, *SGPL1* has been implicated as a mediator of DNA damage response (DDR). Increased *SGPL1* expression and activity in DDR promotes apoptosis through a pathway involving p53 and caspase 2 (30). Therefore, defects in DDR may also contribute to the pathogenesis of *SGPL1* deficiency. However, it should be noted that the T cell defects in patients with *SMARCAL1* mutations are global and involve impairment of T cell proliferation, survival, and function. In contrast, *SGPL1* deficiency in mice leads to lymphopenia as a result of trafficking defects, as shown by the sequestration of mature T cells in mutant mouse thymus, whereas T cell proliferation in thymus is unaffected (J.D. Saba and colleagues, unpublished observations).

Patient fibroblasts had significantly elevated C22:0, C24:0, and C24:1 ceramides compared with control fibroblast (Supplemental Figure 8). Interestingly, total serum ceramide levels and C24:0 and C16:0 lactyosylceramides were found to be high in children with CKD, which suggests they may play a causal role in the development of FSGS in our cohort (31). Total ceramide levels and the balance of different ceramide species can influence apoptosis and autophagy, thereby contributing to the pathophysiology of many diseases.

S1P is a bioactive lipid implicated in the regulation of cell survival, apoptosis, proliferation, and migration (32, 33). It can act in a paracrine and autocrine fashion by acting on G protein–coupled S1PRs that account for activation of signaling pathways such as Akt, mTOR, and RHO/RAC/CDC42 (34). However, S1P can also act intracellularly by regulating proapoptotic effector molecules such as BAK and BAX (35), TRAF2 (36), HDAC activity, and thus, epigenetic programs in the nucleus (37). The pathogenesis of disease within the target organs could result from both an excess of intracellular S1P and an imbalance of other sphingoid bases, but also from S1P signaling through the S1PRs. Podocytes are known to exhibit S1PRs, and as components of the glomerular filtration barrier, they could exhibit increased sensitivity to circulating S1P levels, especially under conditions of reduced *SGPL1* activity, as has been shown in neuron primary cultures (38). In *Drosophila*, the phenotype we observed in nephrocytes upon constitutive *Sply* deletion is most likely to be secondary to intracellular accumulation of S1P, but also to increased interstitial S1P levels, and cannot argue against or in favor of a cell no-autonomous effect of SGPL1 deficiency. However, in our study, cultured podocytes stimulated with S1P did not show increased apoptosis at the S1P expected disease level. Additionally, *SGPL1* knockdown in podocytes also did not lead to a phenotype in cell migration or cell proliferation, although these results could be due to insufficient SGPL1 decrease, since in neurons, a 70% reduction of *SGPL1* using an RNA strategy is insufficient to induce accumulation of S1P (38). In *Drosophila*, there has still been no identification of a S1PR orthologue. However, we cannot exclude the possibility of a *Drosophila* S1PR. Accordingly, the phenotype we observed in nephrocytes upon the constitutive *Sply* knockout cannot argue against or in favor of a cell-nonautonomous effect of SGPL1 deficiency.

However, in mesangial cells, *SGPL1* knockdown induced a decrease of active RAC1 and CDC42 and a phenotype of decreased cell migration, which was partially mitigated by S1PR inhibition. Our findings in RMCs and *Drosophila* nephrocytes suggest that both cell types can be affected by SGPL1 deficiency, but it is not clear which cell type is primary in the pathogenesis. To address this, generation of transgenic mice with glomerular cell type–specific deletion of *Sgpl1* is under way.

Methods

Study participants. We obtained clinical data, blood samples, and skin biopsies from SRNS patients from worldwide sources. The diagnosis of NS was made by nephrologists based on standardized clinical and histologic criteria (39). Renal biopsies were evaluated by pathologists. Additional, clinical data were obtained using a standardized questionnaire (http://www.renalgenes.org).

WES. In consanguineous families, we combined WES with HM, as established previously (40, 41). Genetic regions of homozygosity were plotted across the genome (Figure 1A) (42, 43). Exome capture was performed with the Agilent SureSelect V5 Enrichment Capture Kit. The enriched library was sequenced on an Illumina HiSeq 4000 (100 bases paired end). Variant burden analysis was performed as previously described (44). Sequence reads were mapped against the human reference genome (NCBI build 37/hg19) using CLC Genomics Workbench (version 6.5.1) software (CLC bio). High-throughput exon resequencing was performed largely as described previously (9). For the nonconsanguineous family NCR61 and consanguineous family EB, exome sequencing was performed in 2 (NCR61-1 and NCR61-2) and 1 (EB-1) affected children. The enriched library was sequenced on an Illumina HiSeq 2500 (V4 chemistry, 2 × 125 bases). Variants were called as described (45).

RNA extraction, RT-PCR, and cDNA cloning. Human *SGPL1* full-length cDNA was obtained (clone MGC: 60255 IMAGE: 6150776) and subcloned into pRK5-N-Myc, pDEST69-N-FLAG, or BABE-puro-gateway (Addgene plasmid no. 51070). Mutations were introduced using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

SGPL1 enzymatic activity assay. SGPL1 activity in whole cell extracts of patient fibroblasts and HEK293T cells expressing WT and mutant SGPL1 cDNA constructs was measured by quantifying the amount of hexadecenal formed over time as described (46).

3D protein structure. The crystal structure of human SGPL1 (PDB code 4Q6R) was prepared using the protein preparation wizard in Schrödinger Maestro release 2015.1. (version 10.1). After protein prep-

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aration, the effects of mutations were computationally assessed using the Residue Scanning module in BioLuminate 1.8 (Biologics Suite 2015-1: BioLuminate, version 1.8).

Cell culture and transfection. Human podocytes were provided by M. Saleem (University of Bristol, Bristol, United Kingdom) and maintained at the permissive temperature of 33°C in RPMI + GlutaMAX-I (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS, penicillin (50 IU/ml)/streptomycin (50 μg/ml), and insulin–transferrin–selenium-X. RMCs (ATCC CRL-2573) were maintained in DMEM supplemented with 15% FBS, 0.4 mg/ml G418, and penicillin/streptomycin. *SGPL1*-specific and control scrambled siRNAs were purchased from Dharmacon. siRNAs were transfected into podocytes or RMCs using Lipofectamine RNAiMAX (Invitrogen). The target sequences of siRNAs are in Supplemental Table 1. Human fibroblasts were grown in DMEM supplemented with 15% FBS, penicillin/streptomycin, and nonessential amino acids (Invitrogen). HEK239T cells were used for lentivirus production and transfected by the calcium phosphate precipitation method.

Immunoblotting, immunofluorescence, and GST pulldown assay. Experiments were performed as described previously (47). GST-PAK1 and GST-Rhotekin beads were purchased from Cytoskeleton Inc. Anti-SGPL1 (AF5535, R&D Systems), anti-BiP (ab21685), anti–β-actin (ab6276, Abcam), anti-CD31 (MA3100, Thermo), anti-RAC1 (610650), anti-CDC42 (610928), anti-GM130 (610822, BD Transduction Laboratories), anti-Myc (sc-789), anti-RHOA (sc-418), anti-WT1 (sc-7385, Santa Cruz Biotechnology Inc.), anti-GOLGB1(HPA011008), anti–α-smooth muscle actin (A2547), anti-FLAG (F3165, Sigma-Aldrich), anti-HA (11867423001, Roche), and anti-synaptopodin (20-4694, American Research Products) antibodies were purchased from commercial sources. Immunoblotting was quantified by densitometry using ImageJ software (NIH). For immunofluorescence in *Drosophila* nephrocytes, third instar garland nephrocytes were dissected and fixed for 20 minutes in 4% paraformaldehyde at room temperature. For Kirre stainings, an alternative fixation method was used: nephrocytes were heat fixed during 5 seconds at 90°C in 0.7% NaCl, 0.05% Triton X-100 solution (48). The following primary antibodies were used: anti-Kirre (gift from Karl Fischbach, University of Freiburg) anti-HA, and Alexa Fluor 488 or Cyanine Cy3–conjugated anti–horseradish peroxidase (Jackson Immunoresearch). Nile red and Bodipy 493/503 (Thermo Fisher Scientific) were used to stain lipid droplets. For albumin uptake assay, garland nephrocytes were dissected from third instar larvae in Schneider's medium (Pan-Biotech), incubated for 2.5 minutes with 0.1 mg/ml Alexa Fluor 488–conjugated albumin (Thermo Fisher Scientific) at room temperature, and fixed in 4% PFA for 20 minutes. Confocal images were obtained with a Leica TCS SP8 SMD, and ImageJ (NIH) was used for image analysis.

Migration and proliferation assay. Migration assays were performed using the xCELLigence system (ACEA Biosciences). For migration assays, 48 hours after transfection, 4×10^4 cells were plated with serum-free media in the upper chamber of CIM-plate 16. The lower chambers were filled with 10% FBS for chemoattraction or with serum-free media. For proliferation assays, 4×10^4 cells were plated in the E-plate 16. The data obtained were analyzed with RTCA software (ACEA Biosciences Inc.). Results are presented as the time versus cell index curve.

Yeast strains and plasmids. The following yeast strains were used in this study: BY4741-Euroscarf, MAT*a*, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, dpl1Δ::KanMx,* RH4863, MATα *lcb3Δ*::KanMx *dpl1Δ*::KanMx *ura3 leu2 his4 ade2 bar1,* pLCB3::URA3 (PCR amplified genomic *LCB3* cloned into YCplac33), RH431, and MATα *ura3 leu2 his4 bar1* (49). pRS415-ADH[2] was used to overexpress human *SGPL1* in yeast. pRS415-ADH-SGPL1 was constructed by cloning *SGPL1* in the *Xma*I-*Xho*I sites of pRS415-ADH.

PHS toxicity test. Yeast *dpl1Δ* cells transformed with PRS415-ADH bearing WT or mutant human *SGPL1* coding sequences were cultured overnight in synthetic defined minus leucine medium and spotted onto synthetic defined plates containing ethanol (vehicle control) and PHS (40 μM) as previously described (50).

Synthetic lethality test. RH4863 yeast cells were transformed with plasmids overexpressing human SGPL1 (WT and mutant variants) or yeast (positive control). Cells grown in synthetic defined minus leucine medium were 10-fold serially diluted and spotted onto synthetic defined–agar without leucine, without uracil, or with 1 mg/ml 5-FOA (50).

Fly strains and generation of transgenic flies. Stocks were maintained on standard cornmeal-yeast food at 25°C. The following fly stocks were used: *Sply*^{05091}*/CyO,Act-GFP* (from BL #11393), *Df(2R)BSC433/CyO,Act-GFP* (from BL #24937), and *Df(2R)247/CyO,Act-GFP* (BL #7155), obtained from the Bloomington Stock Center, and *Kirre* RNAi (GD #14476), obtained from the Vienna *Drosophila* RNAi Center. *Pros-GAL4* (a gift from Barry Denholm, Center for Integrative Physiology, University of Edinburgh, Edinburgh, United Kingdom) *y,w*^{1118} was used as a WT control. Due to inbreeding, homozygous *Sply*^{05091} (*Sply* null allele) were almost impossible to obtain. We therefore generated *Sply* null hemizygous flies by crossing the *Sply*^{05091}*/CyO,Act-GFP* flies with *Df(2R)BSC433/ CyO,Act-GFP* flies that carry a deletion of the *Sply* locus and 5 other contiguous genes. In the F1 generation, *Sply* null hemizygous flies were identified by the absence of the balancer chromosome (*CyO,Act-GFP*). We confirmed the absence of *Sply* mRNA in *Sply* null hemizygous flies by reverse-transcriptase PCR (RT-PCR) (Supplemental Figure 15). For *Sply* rescue constructs, the HA tag (in-frame before the stop codon) and the corresponding *SGPL1* human mutations were individually inserted by site-directed mutagenesis in the cloned 4-kb *Sply* genomic locus, including regulating sequences (–491 bp) and 3′ UTR (+1066 bp). Subsequently, the WT and mutant *Sply* rescue constructs were cloned into a *pattB* vector (Konrad Basler, Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland) and injected into flies with an attP landing site at 76A2 by BestGene Inc. Primer sequences and cloning details are available in Supplemental Table 2. For the rescue experiments, 1 copy of the *Sply* rescue constructs was transferred by mating to the *Sply* null hemizygous background.

Viability assay. Fly crosses were allowed to lay eggs for 48 hours on standard cornmeal-yeast food. The percentage of hatching adults of the appropriate genotype was recorded. As a control, we crossed the Df(2R)BSC433/CyO,Act-GFP with Df(2R)247/CyO,Act-GFP (carrying a deletion nonoverlapping with Df[2R]BSC433), and the percentage of the normally viable Df(2R)BSC433/Df(2R)247 flies was recorded. On average, a total of 120 F1 hatching flies were counted per condition, and the experiments were repeated 6 times. Values reported were normalized to the control.

Mice. *Sgpl1*$^{-/-}$ mice were previously described (11). *Sgpl1*$^{-/-}$ mice, littermate controls, and heterozygote breeders were handled in pathogen-free conditions.

TEM. After dissection, third instar garland nephrocytes and kidneys from *Sgpl1*$^{+/+}$ and *Sgpl1*$^{-/-}$ mice were fixed in 2.5% glutaraldehyde at room temperature and processed for TEM with standard techniques.

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Sphingoid bases analysis. Sphingolipid metabolites from fly larvae and patient fibroblast samples were extracted using a protocol described previously (51). Analytical systems used were composed of Agilent 1290 UPLC coupled with Agilent 6490 triple quadruple mass spectrometer. Analysis was carried out using multiple reaction monitoring (MRM) mode. The general source settings in the positive ionization modes were as follows: gas temperature, 200°C; gas flow, 16 $minute^{-1}$; nebulizer, 20 psi; sheath gas temperature, 250°C; sheath gas flow, 11 $minute^{-1}$; and capillary voltage, 3.0 kV. Specific MRM transitions for fly sphingoid bases were programmed based on the transitions described previously (52). Chromatographic separation of sphingolipids was performed with a Zorbax RRHD Eclipse Plus C18 (2.1 × 150 mm, 1.8 μm) equilibrated at 50°C. A binary gradient of mobile phase A (water containing 0.2% formic acid and 2 mM ammonium formate) and B (methanol containing 0.2% formic acid and 1 mM ammonium formate) was delivered at a constant flow rate of 1 ml/min. The total run time was 10 minutes. Initial gradient was 70% B and increased to 100% B at 8 minutes and returned to baseline at 8.5 minutes and maintained until 10 minutes. Data processing of all metabolites was performed using MassHunter software (Agilent).

RT-PCR and real-time PCR. Total RNA was isolated from fibroblasts using a QIAGEN RNA extraction kit (QIAGEN). cDNA was prepared using reverse transcriptase Superscript II (Invitrogen). PCR was performed using ReadyMix Taq PCR (Sigma-Aldrich) and the following primers: SGPL1 exon 4 forward, 5′-CTCACCAGGAAGATGCCCAT-3′ and SGPL1 exon 8 reverse, 5′-GCTTTGCAGGCCATCAGTAT-3′. The amplified PCR products were then cloned into a pGEM–T Easy Vector (Promega) and analyzed by Sanger sequencing. In *Drosophila*, relative expression levels were determined using the Absolute SYBR Green ROX Mix (ABgene) and specific primers as follows: *Sply* forward, 5′-CTGGCTCTGGACTGTGATCTG-3′ and reverse, 5′-GACGCTTGCCACGAATGTAA-3′. Expression levels were normalized to actin.

Statistics. Results are presented as mean ± SEM or SD for the indicated number of experiments. Statistical analysis of continuous data was performed with 2-tailed Student's *t* test or 1-way ANOVA, with Dunnet's, Bonferroni's, or Dunn's post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

Study approval. Approval for human subject research was obtained from the University of Michigan (Ann Arbor, Michigan, USA) and Boston Children's Hospital institutional review boards and the Comité de Protection des Personnes Ile de France II. All subjects gave informed consent. Approval for mouse research was obtained from the University Committee on Use and Care of Animals (UCUCA) of the UCSF Benioff Children's Hospital Oakland and Boston Children's Hospital.

Supplemental appendix. For additional data, including Supplemental Tables 1–3 and Supplemental Figures 1–15, see the supplemental material, available with the full text of this article.

Author contributions

SL, SG, HYG, SS, CES, KS, RPL, OG, MF, MAC, C Antignac, and FH prepared and evaluated genetic mapping and exome sequences. SL, SG, HYG, KS, MAC, C Antignac, and FH identified mutations in the human *SGPL1* gene. SG, HYG, SL, BO, WIC, SH, MZ, VC, C Arrondel, OB, VV, JDS, C Antignac, and FH provided and evaluated critical cell lines and animal resources. JDS, BO, JHS, HYG, and WIC performed all mouse studies and analyzed mutant SGPL1 expression or enzyme activity in fibroblasts and HEK293 cells. JDS, BO, HYG, and WIC analyzed the *Sgpl1*$^{-/-}$ mouse model, critically evaluated mouse data, and wrote mouse studies. SG, N Lachaussée, MS, JP, and C Antignac performed *Drosophila* studies. AS performed TEM. JHS performed all LC/MS assays for quantification of cellular SPLY activity and SPL distribution. HR and VG performed yeast complementation experiments. SL, HYG, SG, MZ, VC, C Antignac, D Schapiro, MF, DAB, FS, NK, JG, TG, KS, VC, D Schanze, IF, YS, AST, BA, JD, RB, YP, EA, N Lloberas, AM, CES, FPB, JPM, VRD, AMC, MCW, MAC, NW, and FH recruited patients and gathered detailed clinical information for the study. JG and TG performed the immunophenotyping of patient EB-1. HS, RW, and GC performed structural molecular modeling of the SGPL1 enzyme. JDS directed studies undertaken by BO and JHS. All authors critically reviewed the paper. FH and C Antignac conceived of and directed the entire project and wrote the paper with help from SL, SG, HYG, BO, and JDS.

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SUPPLEMENTAL APPENDIX

SUPPLEMENTAL ACKNOWLEDGMENTS

We are grateful to Bloomington Stock Center (NIH P40OD018537) for providing the fly strains used in this study and Mar Ruiz Gomez, Centro de Bio-logia Molecular, Madrid, for the nephrocyte heat fixation protocol. This work was performed under the Care4Rare Canada Consortium funded by Genome Canada, the Canadian Institutes of Health Research, the Ontario Genomics Institute, Ontario Research Fund, Genome Quebec and Children's Hospital of Eastern Ontario Research Foundation. Steering committee members and their affiliations of Care4Rare Canada are

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SUPPLEMENTALTABLES AND FIGURES

Supplemental Table 1. Target sequences of siRNAs used in this study.

Human *SGPL1*	
siRNA#5	CAUCAUUGGUGUUGUAUAG
siRNA#6	GAAACGAGUAGCUAUACAA
siRNA#7	CAUUAUUGGUCGUAAGAUU
siRNA#8	GAACGGCUAUGUUGAAGCU
Rat *Sgpl1*	
siRNA#9	CUAAAGGAUAUCCGGGAAU
siRNA#10	AGUUUAUGGUCUCGGUUUA
siRNA#11	GCUCUGGGCUCCAACCGAUU
siRNA#12	GAAUGAAGAUUGUACGCGU

Supplemental Table 2. *Drosophila Sply* locus cloning and mutagenesis primers.

Sply* locus cloning	
Sply Forward 1	TGATCTGGTGGAGCTCGTCACT
Sply Reverse 1	AACCGCTCCACCTTCGCTCA
Sply Forward *2*	GATTTGGCAGCAAGTGTTGGT
Sply Reverse *2*	GAAATCCCTGTACGCCTTCA
Mutagenesis primers	
Sply HA tag Forward	CACTCCCAGCCAGAAATACCCATACGATGTTCCCGATTACGCTTAGACAC CTGGAGC
Sply HA tag Reverse	GCTCCAGGTGTCTAAGCGTAATCGGGAACATCGTATGGGTATTTCTGGCT GGGAGTG
Sply E119G Forward	CCTCCGACTGGTAGATGGGCACCTGAAGACTGG
Sply E119G Reverse	CCAGTCTTCAGGTGCCCATCTACCAGTCGGAGG
Sply R210Q Forward	CCATGAAGGCATACCAGGATTTCGCTAGAGAG
Sply R210Q Reverse	CTCTCTAGCGAAATCCTGGTATGCCTTCATGG
Sply S335I Forward	GGTTAAGGGTGTGACCATTATCTCCGCTGATACC
Sply S335I Reverse	GGTATCAGCGGAGATAATGGTCACACCCTTAACC

* *Sply* locus cloning was performed by amplifying two partially overlapping regions followed by homologous recombination.

Supplemental Table 3. Immunological profile of patient EB-1.

Comprehensive analysis of leukocyte subpopulations by multi-color flow cytometry was performed using the following 4 HIPC panels: T-cell-panel: CD45; CD3; CD4; CD8; CD28; CD38; CD45RA; CD57; CD127; CD183; CD185; CD196; CD197; CD278; CD279; HLA-DR; TCR×™; Treg-panel: CD45; CD25; CD4; CCR4; CD127; CD45R0; CD3; HLA-DR; B-cell-panel: CD10; CD45; CD24; CD19; IgD; CD38; CD20; CD27; CD3; Innate-panel: CD45; CD56; CD123; CD11c; CD16; CD3+CD19+CD20 (dump); CD14; HLA-DR.11 Briefly 100 µl of whole blood was added to the panel tubes with pre-mixed antibodies and incubated for 30 minutes at room temperature in the dark with light agitation. After red cell lysis, cells were fixed and analyzed on a BD Fortessa instrument. A complete and detailed protocol for HIPC immunophenotyping can be downloaded from www.entire-net.eu /sops-and-cats.

Patient EB-1 suffered from severe lymphopenia (218/µl $CD4^+$; 23/µl $CD8^+$; 25/µl γ/δ T cells and 39/µl B cells). Among the $CD4^+$-lymphocytes only 19% of the cells displayed a naïve phenotype, T_{EM} represented 42%, T_{CM} 23% and T_{EMRA} 16% of the cells. In the $CD8^+$- compartment 47% of the cells showed a naïve phenotype, T_{EM} represented 27% and T_{EMRA} 22% of the cells. The few circulating B cells also showed mostly a memory phenotype. Remarkably 36% of the circulating B cells were plasmablasts. The number of regulatory T cells was in the normal range, the same for NK cells, however the proportion of $CD56^{br}CD16^+$ was elevated. Monocytes were in the normal range, the ratio of myeloid to plasmacytoid DC's was 2:1. Functional analysis was performed using gene expression analysis after whole blood LPS and PMA/ionomycin stimulation. After LPS stimulation the expression of pro-inflammatory genes was decreased in comparison to the reference group. After P/I stimulation the expression of most cytokines was decreased, perhaps due to the low T cell count. The expression of TNFSF13B (BAFF) was elevated both spontaneously and after stimulation.

Flow-cytometry

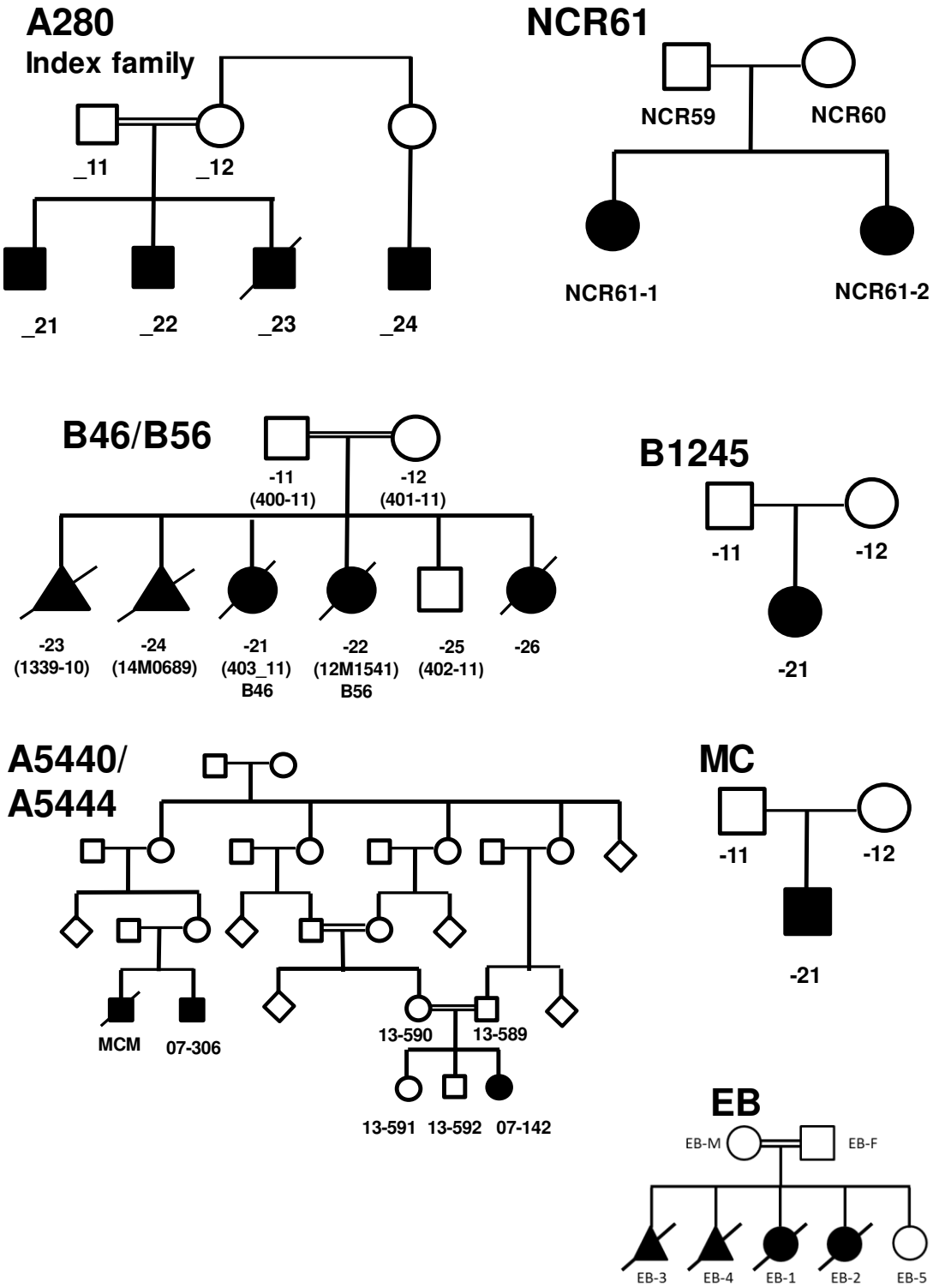
T cells				B cells	NK cells	Monocytes	Dendritic cells
CD4+	**CD8+**	**γδ T cells**	**Treg**				
218/µL ⇓⇓⇓⇓ (1400-5200/µl) Naïve: 19%# T_{EM}: 42% T_{CM}: 23% T_{EMRA}: 16%	23/µL ⇓⇓⇓⇓ (600-3000/µl) Naïve:47%# T_{EM}: 27% T_{EMRA}: 22%	25/µl (17-221/µl)*	65/µl (56-104/µl)*	39/µl ⇓⇓⇓⇓ (500-3600/µl) 72% memory cells## 36% plasmablasts	376/µl (200-1800/µl)	725/µl (700-1500/µl)	228/µl (29-156/µl)* Myeloid to plasmacytoid DC ratio 2:1 (6:1)*

Functional analysis

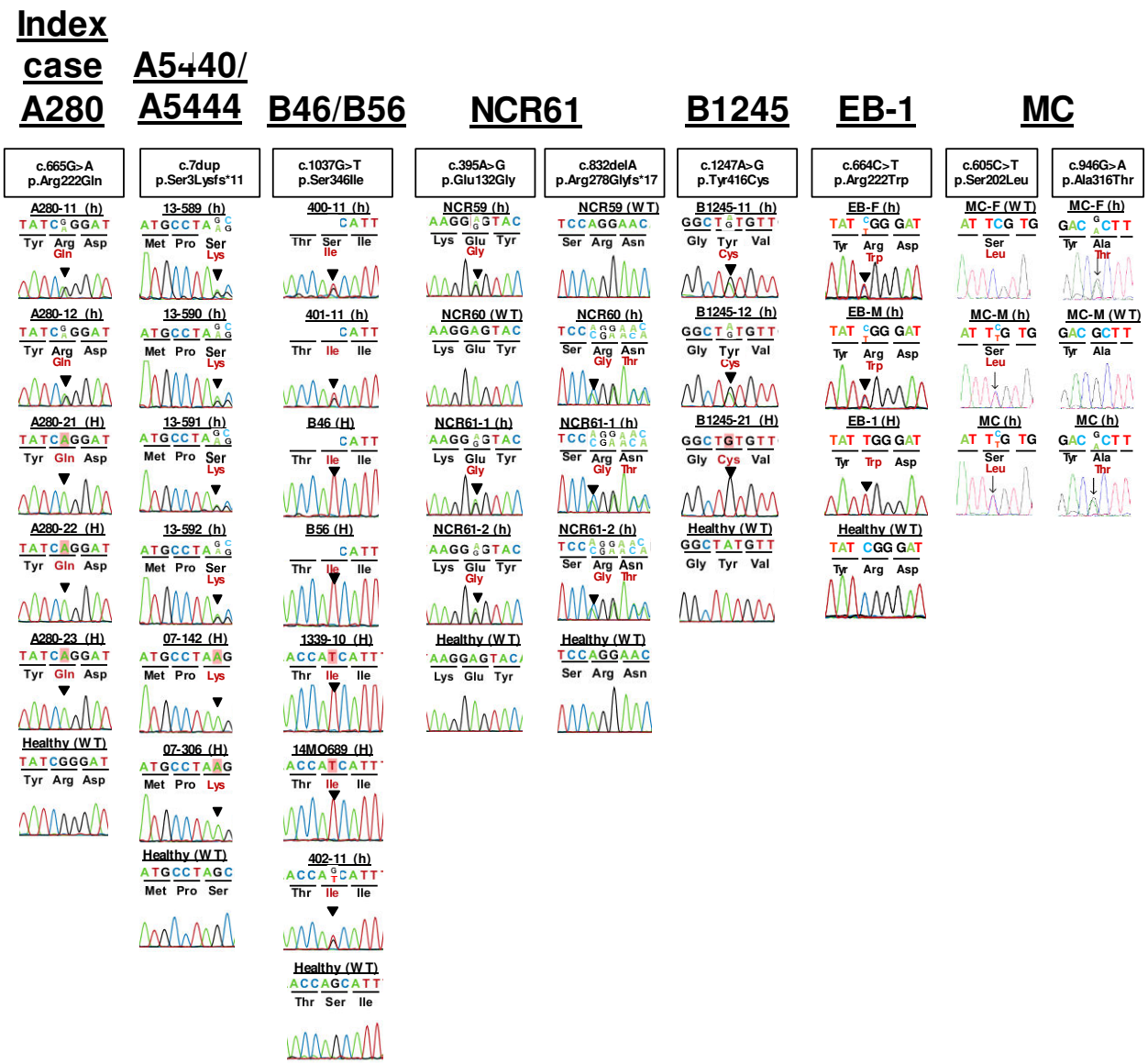
	Spontaneous		LPS		PMA/Ionomycin	
	EB-1	**Reference**	**EB-1**	**Reference**	**EB-1**	**Reference**
IFNgamma	2**	9 ± 15	8	122 ± 142	7014	122576 ± 50241
IL1ß	486	2035 ± 1885	5481	26377 ± 8366	2438	3694 ± 1616
IL2	3	1 ± 1	12	1 ± 1	5649	103217 ± 43349
IL4	4	4 ± 3	0	8 ± 4	412	2101 ± 1143
IL6	2	9 ± 9	590	6568 ± 4057	72	423 ± 270
IL8	4832	6921 ± 6511	9939	20984 ± 11090	364760	75382 ± 53044
IL10	2	3 ± 1	12	13 ± 7	21	1046 ± 645
IL12-p40	0	0 ± 0	0	141 ± 142	1	0 ± 0
IL17	0	0 ± 0	0	0 ± 0	42	3526 ± 1496
IL22	0	0 ± 0	0	1 ± 1	64	4143 ± 2068
TNFalpha	27	175 ± 172	92	965 ± 686	627	6573 ± 4669
TNFSF13B	1681	260 ± 152	1607	544 ± 408	482	38 ± 37

T EM- Effector memory T cells; TCM- Central memory TEMRA- Effector memory RA T cells; *range not age-matched; # naive T cells constitute usually >80% of the T cells in this age group; ## memory B cells constitute usually <6% of the B cells in this age group **Transcripts / 1000 transcripts PPIB

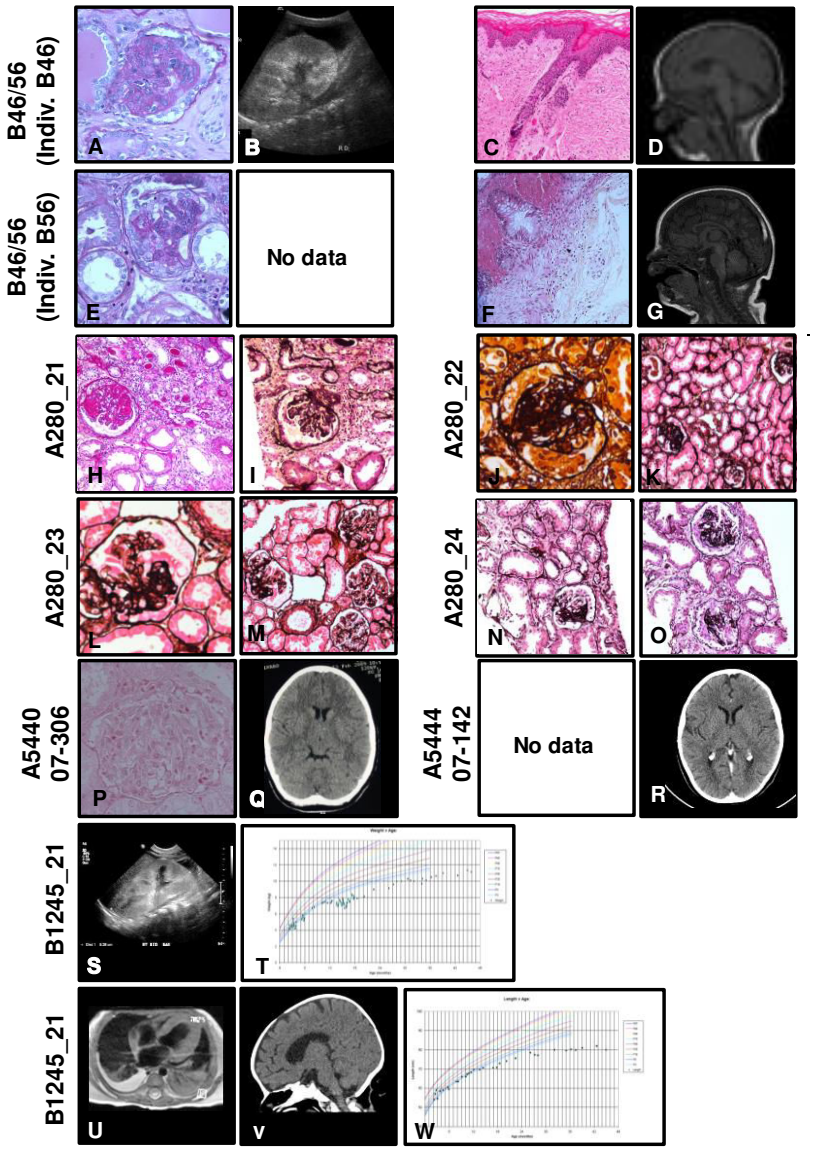
SUPPLEMENTAL FIGURES



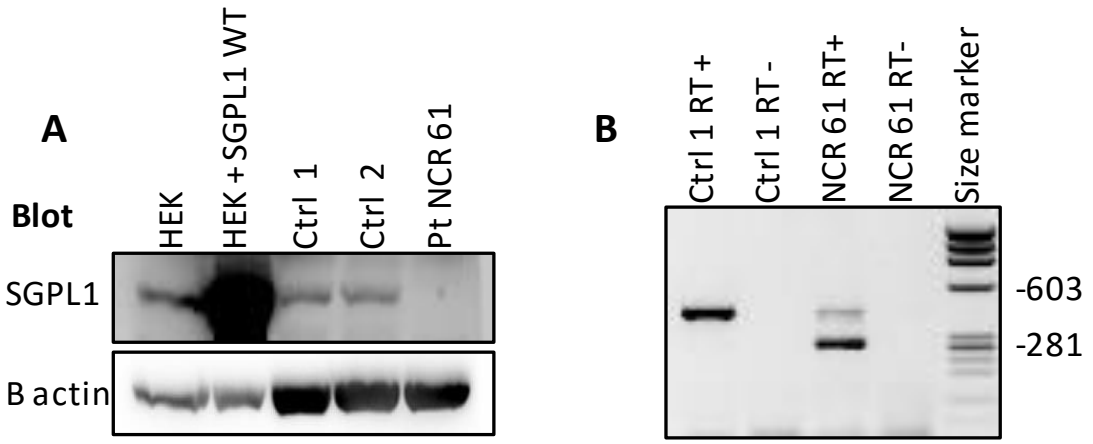
Supplemental Figure 1. Pedigrees of 7 families with *SGPL1* mutations.
Pedigrees of Family A280, NCR 61, B46/B56, A5440/A54444, MC, EB and B1245 are shown. Squares indicate male family members, circles female family members, triangles unknown gender, slashes deceased individuals, solid symbols affected family members and double horizontal bars consanguinity. Numbers below circles and squares indicate the sibling number. Symbols without numbers indicates that DNA was not available for these individuals.



Supplemental Figure. 2. Sequencing traces of individuals with mutation in *SGPL1*.
Sequencing traces are shown for individuals from 5 families with mutations in *SGPL1* for families A280, A5440/A5444, B46/B56, NCR61, B1245, EB and MC. Arrowheads denote altered nucelotides.



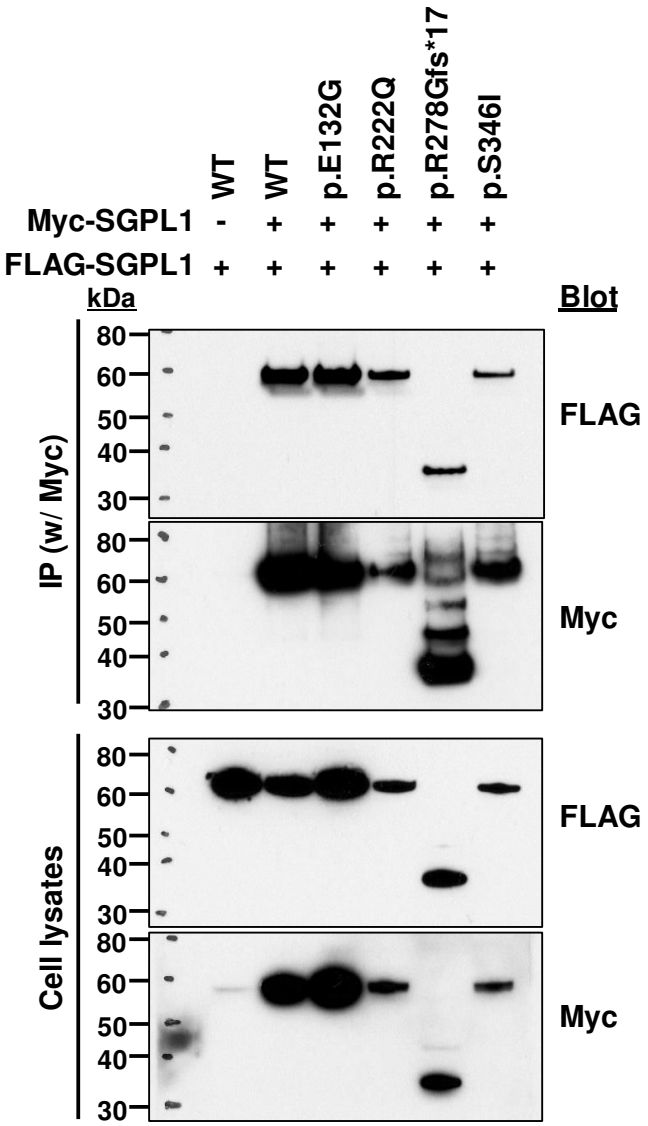
Supplemental Figure 3. Additional clinical phenotypes of individuals with mutations in *SGPL1*. Family and individual numbers are shown per row. **(A)** Renal histology in individual B46 showing diffuse mesangial sclerosis (DMS). **(B)** Renal ultrasound in B46. **(C)** Skin biopsy in B46 showing a moderate degree of hyperkeratosis with a reduced and focally absent granular layer. Infundibula of hair follicles are dilated with keratotic follicular plugs. The dermis and cell-rich stroma are normal. No signs of inflammation, or vacuolar/granular degeneration of keratinocytes. Image is considered characteristic for ichthyosis vulgaris. **(D)** Cranial MRI of B46 showing microcephaly. **(E)** Renal histology of B56 showing DMS. **(F)** Skin biopsy of B56 showing ichthyosis vulgaris. **(G)** Cranial MRI of B56 showing microcephaly. **(H, I)** Renal histology of A280_21 showing global sclerosis and focal segmental glomerulosclerosis (FSGS). (J, K) Renal histology of A280_22 with FSGS (silver methenamine staining). **(L, M)** Renal histology of A280_23 with limited tubular atrophy, expanded mesangium and global and segmental glomerulosclerosis. **(N, O)** Renal histology of A280_24 showing FSGS. **(P)** Renal histology images of A5440/07-306 individual with mutations in SGPL1 showing focal-segmental glomerular sclerosis. **(Q)** Brain CT scan in individual A5440/07-306, microcephaly is not present **(R)** Brain CT scan in individual A5444/07-142, microcephaly is not present. **(S)** Renal sonography in individual B1245_21. **(T)** Body weight percentiles for B1245_21 with failure to thrive. **(U)** Cardiac MR of B1245_21 with reduced left ventricular systolic function (EF~50%) and reduced diastolic function. **(V)** Brain CT scan of B1245_21. Microcephaly is not present. **(W)** Height percentiles for B1245_21 with failure to thrive with height <3rd%.



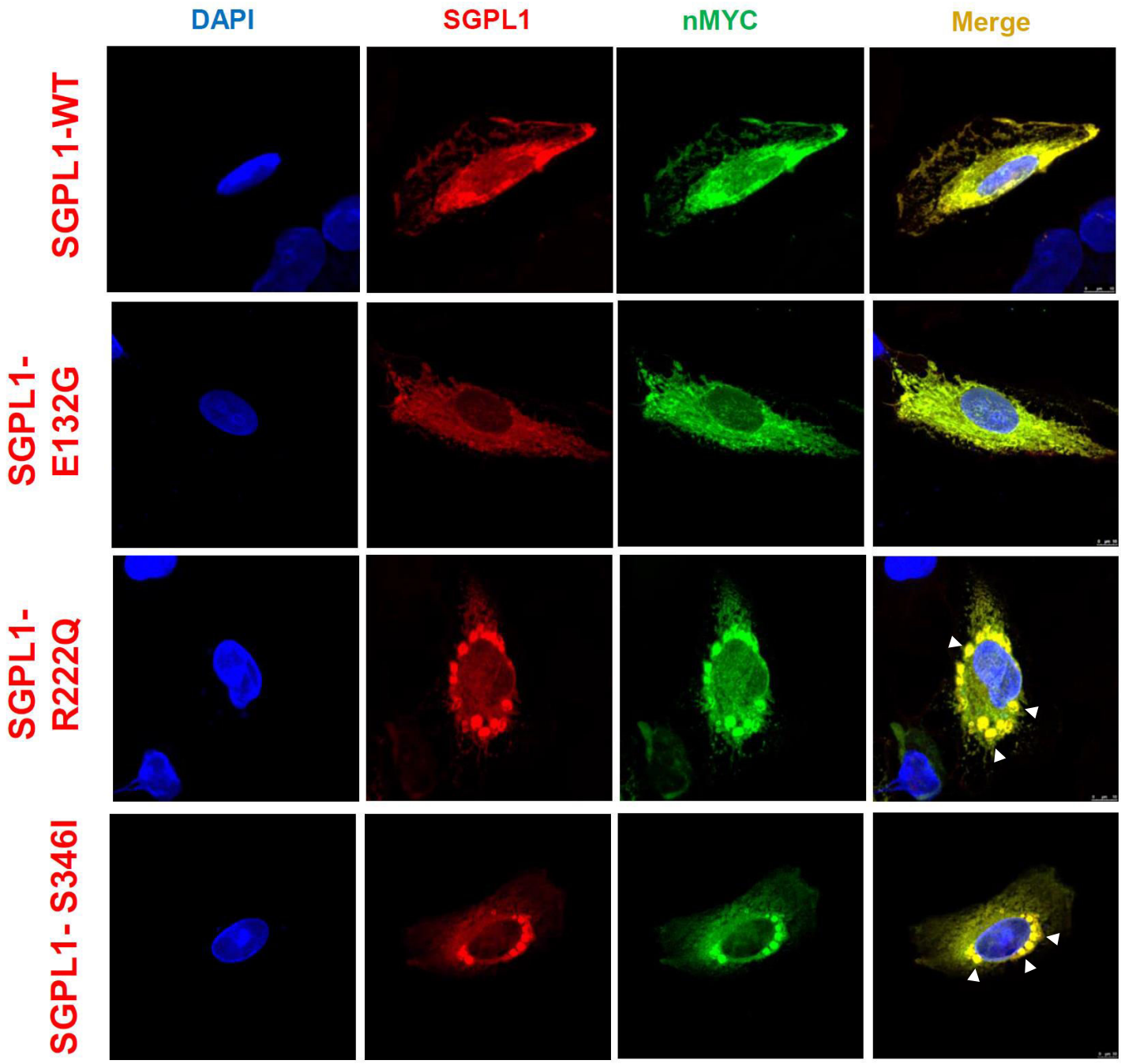
Supplemental Figure 4. Immunoblot in patient fibroblasts and consequence of *SGPL1* splice site mutation c.395A>G.

(A) SGPL1 protein is absent from NCR61 patient fibroblasts. While SGPL1 is present in control fibroblasts it is absent from patient NCR61. HEK 293 cells were transfected with SGPL1 WT as a control for SGPL1 antibody. n=3 independent experiments

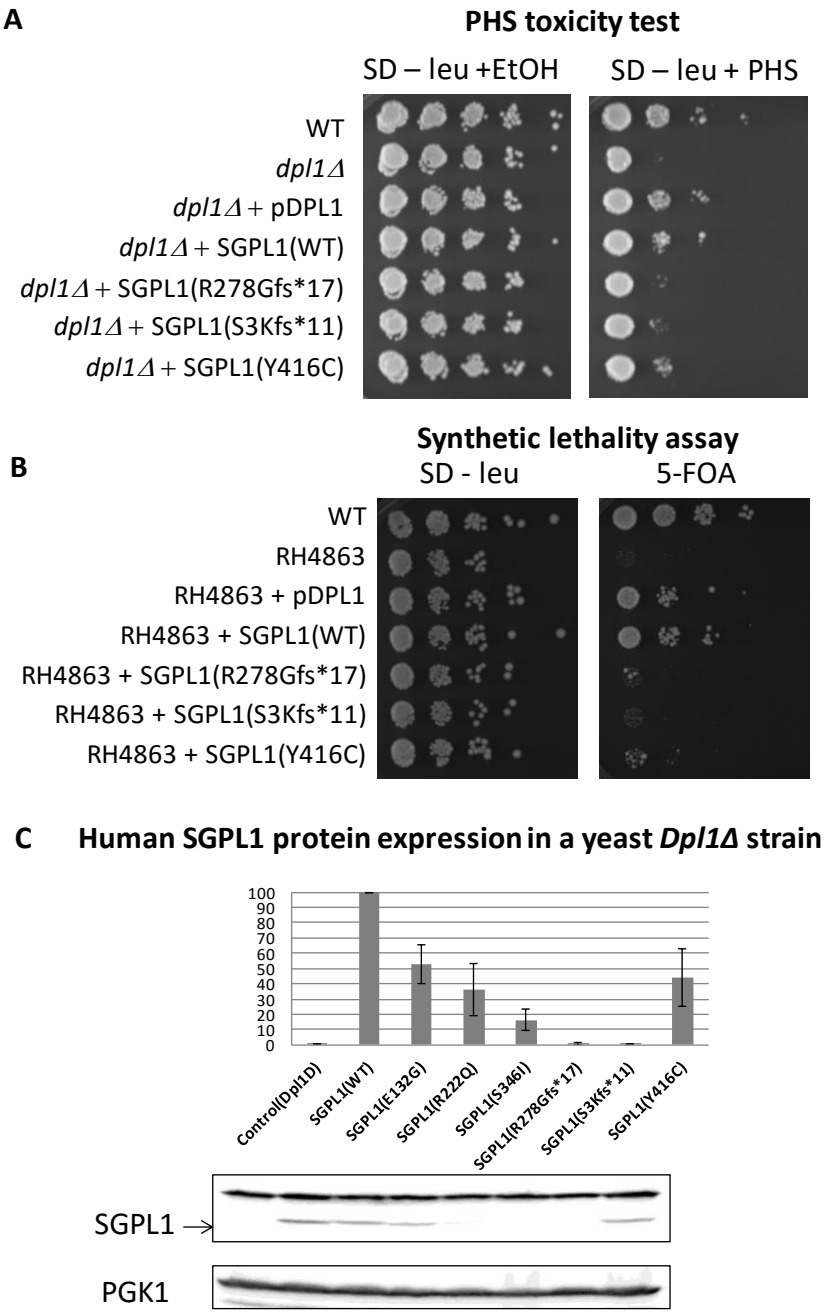
(B) SGPL1 shows splicing defects in NCR61 patient fibroblasts. Reverse transcriptase of mRNA from control and patient NCR61 fibroblasts was performed, followed by PCR amplification of SGPL1 exon 4 to 8, cloning of the amplicons and sequencing. Note the amplification of a light band at the expected size that corresponds to the frameshift allele (c.832delA) and a lower size amplicon corresponding to the loss of exon 5 due to the c.395A>G mutation from the other allele. Sequencing of the c.395A>G product revealed the resulting truncating allele p.Ile88Thrfs*25.



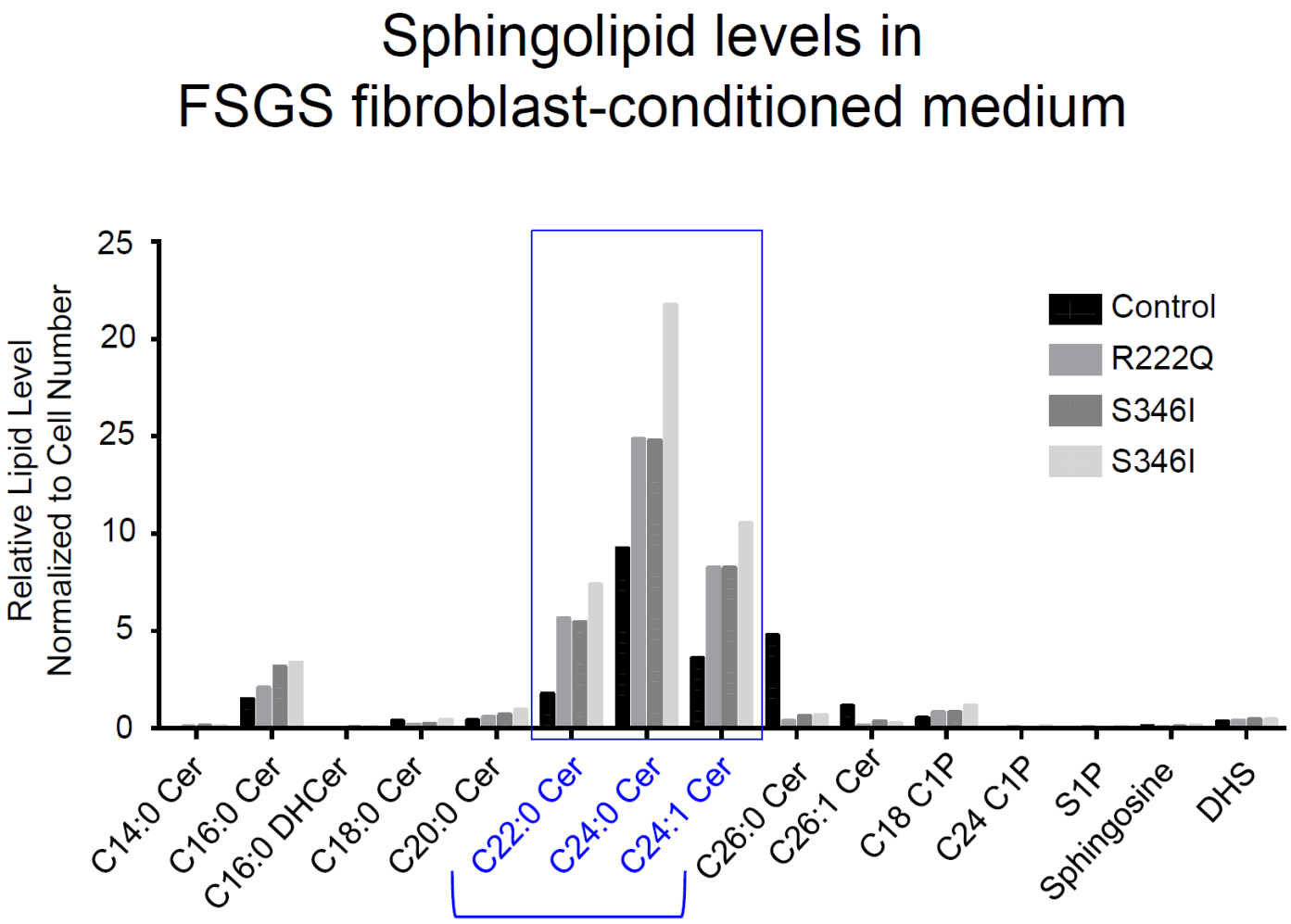
Supplemental Figure 5. Co-immunoprecipitation to assess dimerization of wild type (WT) and mutant SGPL1 protein. Reciprocal experiment to **Figure 2I**. HEK293T cells were transfected with Myc- and FLAG-tagged SGPL1 WT and mutant plasmids. Cell lysates were coimmunoprecipitated with anti-Myc antibody and blotted with anti-FLAG antibody to examine the dimers of WT or mutant SGPL1 proteins. Note that, while all proteins, including the truncated protein (p.Arg278Glyfs*17), still form dimers, p.Arg222Gln and p.Ser346Ile mutant proteins exhibit lower expression levels compared to WT protein upon overexpression in HEK293T cells (see **also Figure 2I**). Coimmunoprecipitation is representative of 3 experiments.



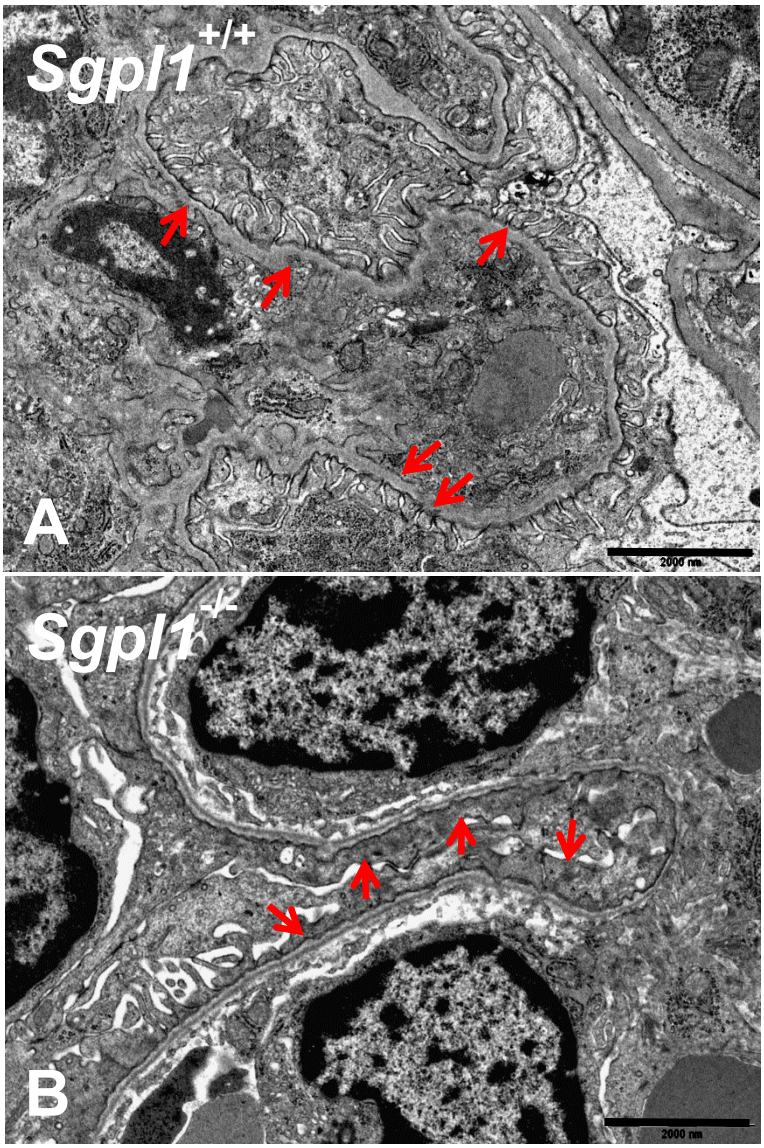
Supplemental Figure 6. Immunofluorescence of *SGPL1* cDNA clones representing wild type (WT) and mutant cDNA clones overexpressed in human immortalized undifferentiated podocytes. Podocytes were transfected with Myc-tagged wild-type (WT) and mutant clones of *SGPL1* detected in patients with nephrotic syndrome. Cells were fixed with cold acetone and coimmunostained with anti-SGPL1 (R&D Systems) (red) and anti-Myc antibody (green). Note that overexpressed p.R222Q and p.S346I variant proteins formed aggregates (white arrow heads) upon overexpression, whereas wild type and missense control p.E132G SGPL1 proteins did not show cellular aggregates.



Supplemental Figure 7. Human *SGPL1* mutations fail to rescue yeast growth.
(A) Phytosphingosine toxicity test. Ability to complement *dpl1Δ* deletion on a medium containing phytosphingosine (PHS) was tested for human SGPL1 WT and the mutants Y416C, R278Gfs*17, S3Kfs*11. None of this mutants was able to restore the impaired growth of a dpl1 knockout strain.
(B) Synthetic lethality test. Synthetically lethal yeast strain RH4863, in which *DPL1* and *LCB3* were deleted, was maintained by expressing *LCB3* from a *URA3*-plasmid. Human WT SGPL1 expressing RH4863 survived on 5-FoA plates, showing to be able to compensate for the loss of *LCB3* and to degrade long-chain bases. However, overexpression of the mutants Y416C, R278Gfs*17, S3Kfs*11, observed in patients with NPHS type 14, did not allow for survival of *DPL1* (*SGPL1*) deficient strains.
(C) Human SGPL1 WT and mutants expression in a yeast *Dpl1Δ* strain. Protein expression of SGPL1 WT and mutants Glu132Gly, Arg222Gln, Ser346Ile, Arg278Glyfs*17, Ser3Lysfs*11, and Tyr416Cys in yeast Dpl1Δ strain. PGK1 was used as a loading control. Values correspond to 3 independent experiments and are shown as % to WT.



Supplemental Figure 8. Sphingolipid levels in fibroblast-conditioned medium. Sphingolipids were extracted from control and patient fibroblast-conditioned medium after 24h conditioning and analyzed by LC/MS, as described in Methods.

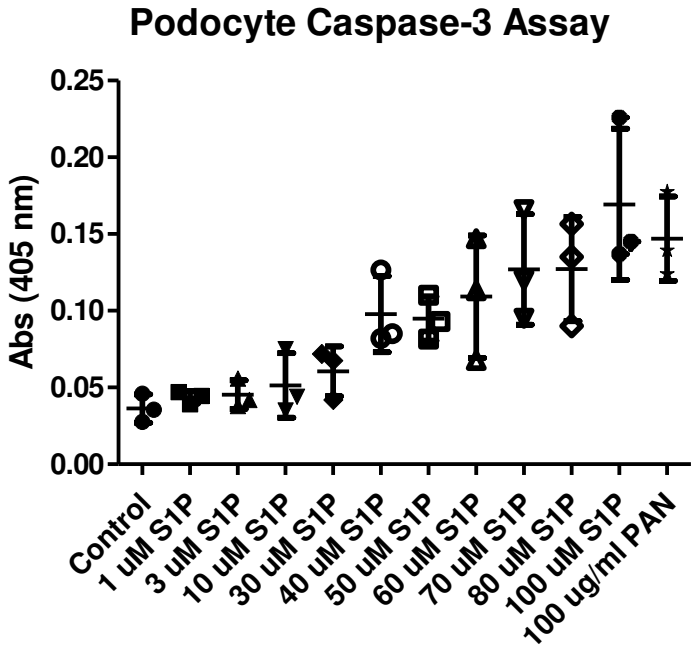


Supplemental Figure 9. Loss of *Sgpl1* causes foot process effacement.

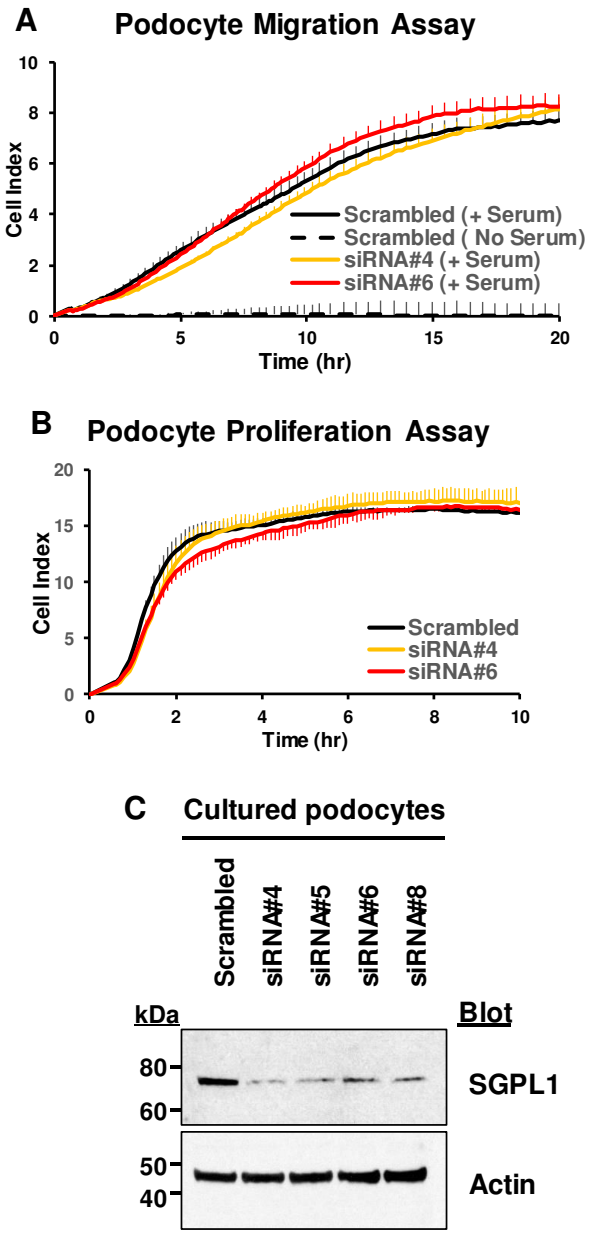
Kidneys of 13 day old *Sgpl1*$^{+/+}$ and *Sgpl1*$^{-/-}$ mice were collected and renal morphology was examined by transmission electron microscopy (TEM).

(**A**) Podocyte foot processes (red arrows) are regularly spaced in kidneys of *Sgpl1*$^{+/+}$ mice.

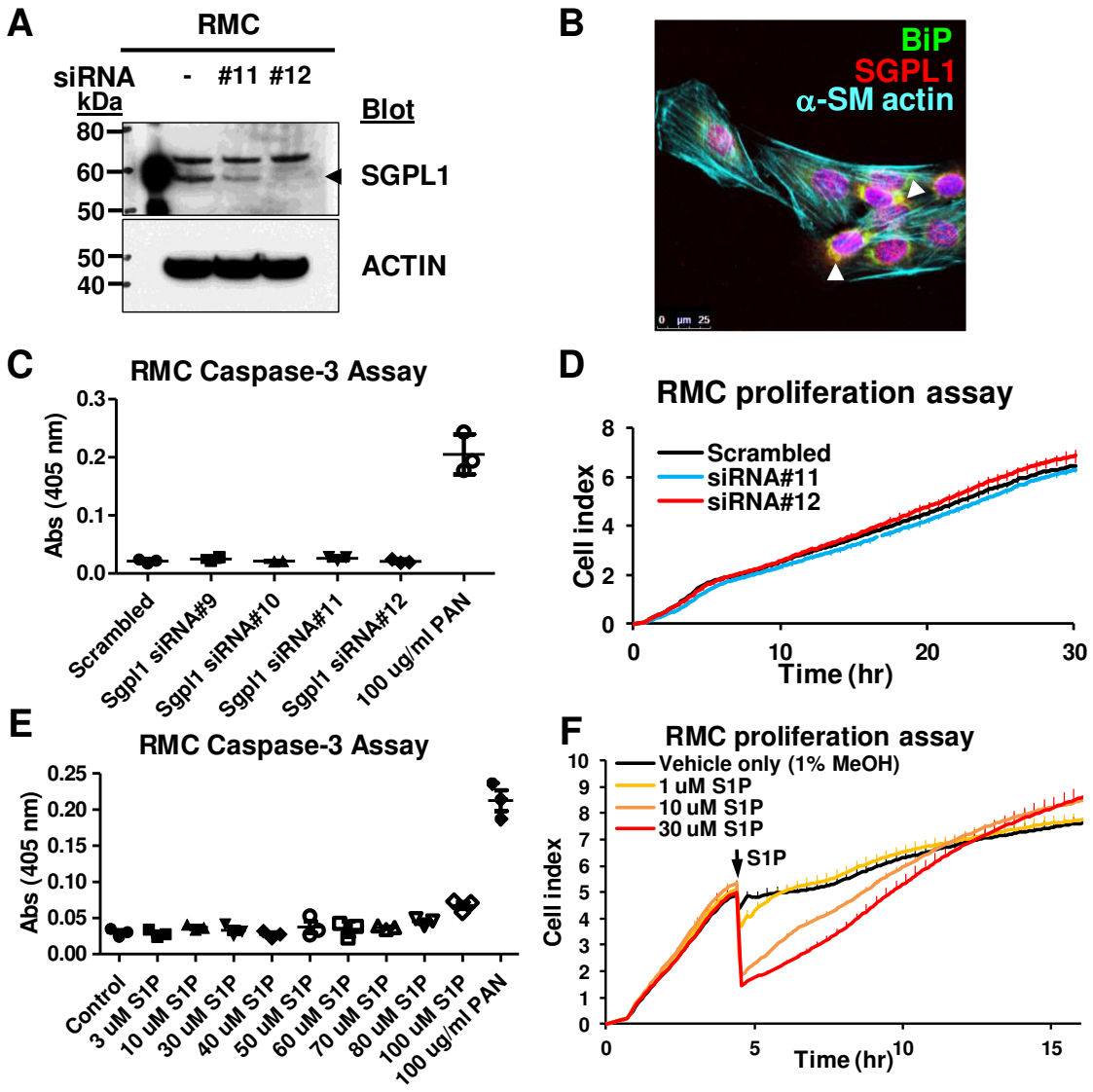
(**B**) *Sgpl1*$^{-/-}$ kidneys exhibit complete foot process effacement (red arrows) and absence of slit-diaphragms. Scale bars are 2 μm.



Supplemental Figure 10. Effect of sphingosine-1-phosphate (S1P) on apoptosis of cultured human podoyctes. S1P or puromycin aminonucleoside (PAN) was incubated for 24 hours and apoptosis was measured by caspase-3 assay. More than 40 μM of S1P induced significant apoptosis in cultured human podocytes. Data represent the mean ± standard deviation of three independent experiments.



Supplemental Figure 11. Effects of *SGPL1* knockdown on migration and proliferation of cultured human podocytes.
(A) *SGPL1* knockdown using two different siRNAs did not have any effect on migration in undifferentiated cultured human podocytes.
(B) *SGPL1* knockdown did not affect proliferation of culture podocytes. Error bars in **A** and **B** are shown in one direction only for clarity and indicate SDs for 3 indipendent expreiments.
(C) The efficiency of *SGPL1* knockdown by 4 different siRNAs was confirmed by immunoblotting. Immunoblot is representative of 3 experiments.



Supplemental Figure 12. Effects of *Sgpl1* knockdown and S1P administration on rat renal glomerular mesangial cells (RMCs).

(A) *Sgpl1* knockdown by siRNAs (siRNA#11 and siRNA#12) in RMCs significantly reduced SGPL1 protein. Immunoblot is representative of 3 experiments.

(B) Immunofluorescence of SGPL1 in RMCs. BiP (green), SGPL1 (red), and α-smooth muscle (SM) actin (sky blue).

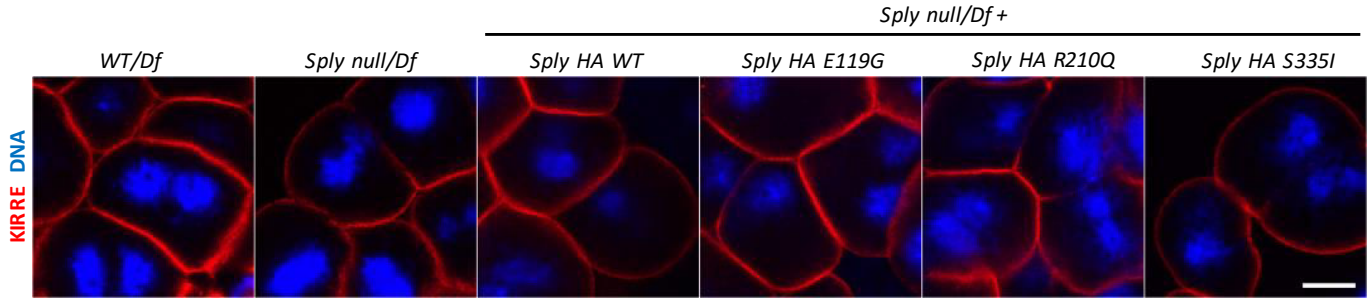
(C) Knockdown of *Sgpl1* in RMCs did not induce apoptosis as measured by caspase-3 assay. D PAN, puromycin aminonucleoside.

(D) Knockdown of *Sgpl1* in RMCs did not affect cell proliferation rate using the xCELLigence system.

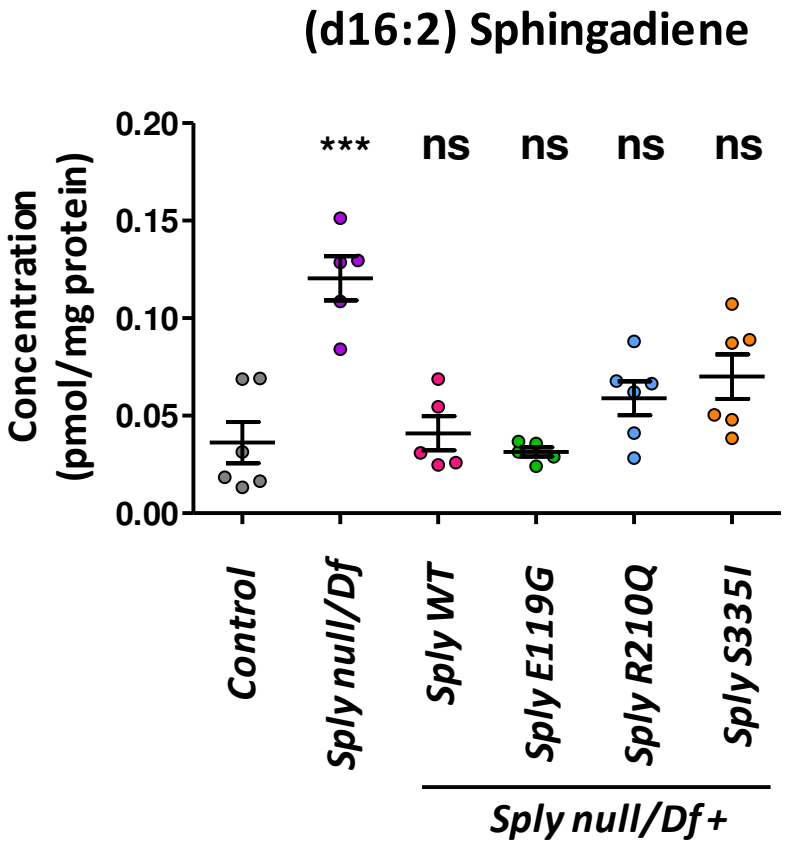
(E-F) Effect of sphingosine-1-phosphate (S1P) on apoptosis (**E**) or proliferation rate (**F**) of RMCs.

(E) Sphingosine-1-phosphate (S1P) administration did not significantly induce apoptosis in RMCs up to 100 μM. S1P or PAN was incubated for 24 hrs. Data represent the mean ± standard deviation of three independent experiments in **C** and **E**.

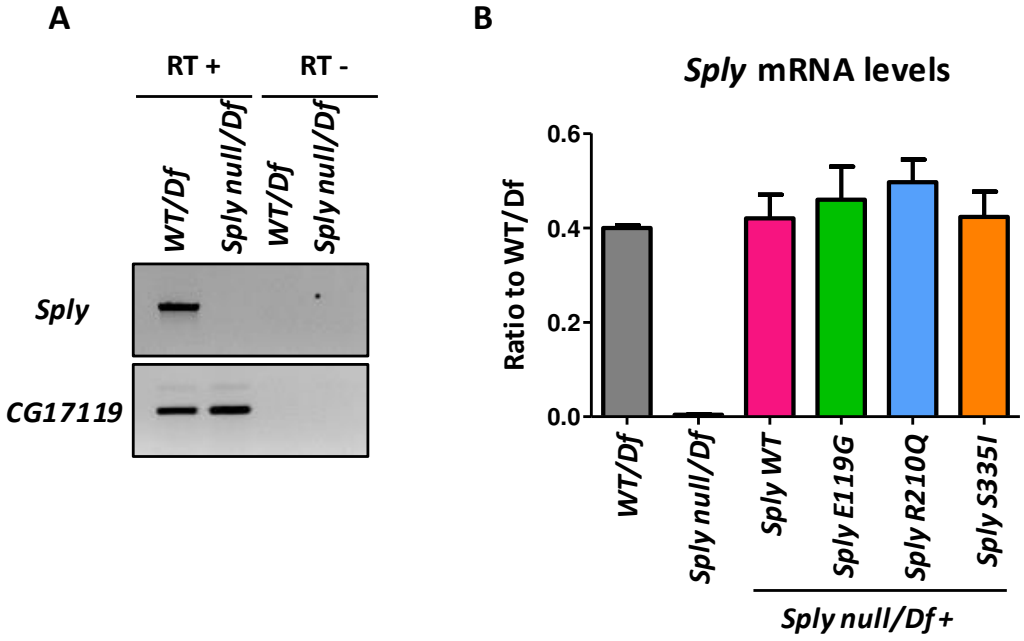
(F) S1P did not induce defects in proliferation of RMCs even though there was a transient drop in cell index with recovery upon addition of high concentration (≥ 10 μM) of S1P. Each cell index value corresponds to the average of more than triplicates in **D** and **F**.



Supplemental Figure 13. *Sply* null hemizygous nephrocytes and SGPL1 mutations do not have any obvious impact on *Kirre* (*Neph1*) expression in nephrocytes.
Immunofluorescence of 3rd instar Garland nephrocytes, stained for the Neph1 ortholog, Kirre (red). No major changes on Kirre staining were observed in the different mutants. n=2 independent experiments (≥5 larvae per genotype). Nuclei were labeled with Hoechst (blue). Scale bar, 10 μm.



Supplemental Figure. 14. Accumulation of sphingadienes (SD) in *Sply null hemizygous* flies. *Sply null* hemizygous flies accumulate sphingadienes and this is rescued by *Sply* WT and E119G mutants. There is a trend towards accumulation of sphingadienes in the mutants R210Q and S335I, although this does not reach statistical significance. *Whole 3rd instar* larvae were collected and processed for LC/MS. (n= 6 independent experiments; for *Sply* null, *Sply* WT and *Sply* E119G, one analysis per group was removed due to poor quality of the chromatography). Control corresponds to WT larvae. Asterisks denote groups that are statistically different from control identified by one-way Analysis of Variance analysis. ***, $p<0.0005$; ns, not significant.



Supplemental Figure 15. *Sply* mRNA levels in *Drosophila*.

(A) *Sply* RT-PCR of 3rd instar larvae heterozygous for the *Df(2R)BSC433* (control) or *Sply null* hemizygous. Note the absence of *Sply* cDNA in *Sply null* hemizygous flies. CG1719 transcript was amplified as a control. n=2 independent experiments.

(B) *Sply* mRNA levels are not different between the *Sply* WT and the mutant rescue constructs. *Sply* qPCR of 3rd instar larvae heterozygous for the *Df(2R)BSC433* (control), *Sply null* hemizygous, or the rescue animals carrying the Sply transgenes WT, E119G, R210Q or S335I on the *Sply null* hemizygous background. n=3 independent experiments.

Summary of the findings and discussion

In this project we identified in 7 families 9 different mutations in *SGPL1* as a cause of a new autosomal recessive syndrome characterized by SRNS, adrenal insufficiency, ichthyosis, immune deficiency and neurological defects. *SGPL1* encodes sphingosine 1-phosphate lyase (SGPL1), a type III transmembrane protein that resides in the ER and is responsible for the irreversible degradation of sphingosine-1-phosphate (S1P) into phosphoethanolamine and hexadecenal (Ikeda, Kihara et al. 2004). This widely expressed protein has a short N-terminal intra-luminal ER domain, a transmembrane domain and a large cytoplasmic pyridoxal-dependent catalytic domain. SGPL1 is the terminal enzyme in the catabolic pathway of sphingolipids, controlling the abundance of the upstream ceramides, sphingosine and S1P (Aguilar and Saba 2012). Most of the identified *SGPL1* mutations were missense mutations located in the region encoding the cytoplasmic catalytic domain of SGPL1.

Through functional studies we and our collaborators have shown that all analyzed mutations decreased the protein expression and *in vitro* lead to a complete or partial loss of SGPL1 enzymatic activity. In collaboration, J. Saba group also found that patient fibroblasts produced an increased amount of ceramide species compared to controls. Moreover, overexpression of the mutated proteins in HEK cells led to formation of cytoplasmic aggregates. Using the yeast model our collaborators (H. Riezman group) showed that although the human SGPL1 is able to rescue the loss of dpl1, the ortholog of *SGPL1*, this was not the case for the mutant proteins. In mouse kidney samples the group of F. Hildebrandt found that SGPL1 was expressed in podocytes, but also in mesangial and endothelial cells and tubules. *In vitro* studies performed in WT human podocytes and in mesangial cell lines did not reveal increased apoptosis upon stimulation with S1P. Moreover, proliferation and migration were not affected by silencing of SGPL1 in podocytes, however in mesangial cells there was a significant decrease in migration that was shown to be mediated by S1PR1 and S1PR3. SGPL1 deficiency also induced decreased activation of Cdc42 and Rac1. Finally using the *Drosophila* model we showed that the *SGPL1* ortholog, *Sply*, is expressed in *Drosophila* nephrocytes and that *Sply* deficiency induced reduced viability, nephrocyte defects and accumulation of sphingoid bases that were rescued by the WT *Sply* but not by the disease-associated variants.

Synchronously, two other groups have also described overlapping phenotypic manifestations in patients harboring biallelic mutations in *SGPL1*. Prasad et al. described 5 families with 8 affected individuals. Interestingly, in this paper some patients presented with adrenal insufficiency without SRNS (Prasad, Hadjidemetriou et al. 2017). Janecke et al. described 3 affected individuals with congenital SRNS, adrenal insufficiency and hypogonadism (Janecke, Xu et al. 2017). Surprisingly,

Atkinson et al. described two siblings with heterozygous compound mutations (a missense mutation and an early truncating mutation) leading to reduced SGPL1 expression, but the only manifestation of the disease was isolated predominantly motor peripheral neuropathy and no other manifestation at 32 and 28 years of age (Atkinson, Nikodinovic Glumac et al. 2017). Interestingly peripheral neuropathy was also described in two unrelated patients in our cohort. These papers confirm the causality of *SGPL1* mutations in a new hereditary syndrome; however, they also show the extreme phenotypic variability of this disease.

Interestingly, *Sgpl1* KO mice phenocopy most of the disease manifestations. These mice exhibit a severe phenotype with growth retardation and death after weaning (Schmahl, Raymond et al. 2007, Vogel, Donoviel et al. 2009). Multiple organ defects are observed, namely in the kidney, cardiovascular system, skeleton, skin, lung and nervous system (Schmahl, Raymond et al. 2007, Vogel, Donoviel et al. 2009, Hagen, Hans et al. 2011, Schumann, Grevot et al. 2015, Mitroi, Deutschmann et al. 2016). Kidney lesions are characterized by FSGS with podocyte foot process effacement, proteinuria and progressive decline in renal function (Schmahl, Raymond et al. 2007, Schumann, Grevot et al. 2015), as observed in humans. Additionally there is lymphopenia, related to impaired trafficking of lymphocytes due to the disruption of the S1P gradient between lymphoid tissues and the blood/lymph (Vogel, Donoviel et al. 2009). Although adrenal disease had not previously been identified in these mice, Prasad et al. clearly showed morphological defects in the cortical zone of the adrenal glands and reduction of some steroidogenic enzymes (Prasad, Hadjidemetriou et al. 2017), and adrenal failure could be a main cause of death in these mice.

Phenotypic variability

Patients with *SGPL1* mutations exhibit large phenotypic variability. The age of onset of the disease can vary widely, ranging from congenital forms to manifestation in young adults. Organ involvement is also highly variable, from one single defect, such as isolated axonal peripheral neuropathy (Atkinson, Nikodinovic Glumac et al. 2017) to severe multiple organ involvement, including the adrenal gland, kidney, thyroid, skin, immune system, nervous system and even the skeleton and rarely the heart (Prasad, Hadjidemetriou et al. 2017). Cases of fetal demise with hydrops fetalis were also present.

This phenotypic variability may be partially explained by the type of mutations and the residual enzymatic activity of SGPL1. This is actually the case in rodent models where the number of organs involved and the severity of the phenotype seem to be highly dependent on the remaining level of

enzymatic activity, with 10% to 20% being enough to suppress most of the disease manifestations (Vogel, Donoviel et al. 2009, Schumann, Grevot et al. 2015). Interestingly, our group and Atkinson et al. report decreased SGPL1 protein levels for the analyzed mutations, in patient fibroblasts or lymphoblasts (Atkinson, Nikodinovic Glumac et al. 2017). Atkinson et al. showed that this was due to either nonsense mediated mRNA decay for a nonsense mutation or to increased proteasomal degradation for a missense mutation (Atkinson, Nikodinovic Glumac et al. 2017).

Our group also analyzed SGPL1 enzymatic activity in patient fibroblasts, and found it to be decreased in all cases (n=3 unrelated patients). Prasad et al. analyzed the protein levels and enzymatic activity of SGPL1 mutations (R222Q or F545del) overexpressed in *Sgpl1* -/- MEFs, and both were found to be significantly reduced (Prasad, Hadjidemetriou et al. 2017). However, due to the different methods and cellular types used in each paper and the low number of mutations analyzed it is difficult to conclude if more severe disease manifestations are related to lower protein levels and enzymatic activity. It would thus be interesting to consecutively test the protein levels and enzymatic activity of SGPL1 in all the patients that carry different mutations, for example using blood lymphocytes, to see if this is the main factor modulating the disease manifestations. However the sensitivity of the different methods should be addressed first in order to see if they can distinguish between small differences in SGPL1 levels or enzymatic activity.

Supporting the main role of the mutation pathogenicity in patient phenotype we showed that the disease severity in humans was highly reproducible for the mutations S346I and R222Q in the *Drosophila* model. In *Drosophila* the deficiency of the *SGPL1* ortholog, *Sply*, induces reduced viability, increased apoptosis in embryos, muscle defects and decreased egg laying (Herr, Fyrst et al. 2003), as well as accumulation of sphingoid bases (Herr, Fyrst et al. 2003, Fyrst, Zhang et al. 2008), and we have also shown that these flies display nephrocyte defects. In humans, patients with the S346I homozygous mutation had a far earlier onset of the disease and more extensive organ involvement in humans, than the R222Q homozygous mutations. Concordantly, *Sply null* flies carrying the corresponding S346I rescue construct had a more severe phenotype than the ones carrying the R222Q rescue construct, showing higher lethality, more significant nephrocyte defects and increased accumulation of sphingosine and ceramide species in 3rd instar larvae. Thus, in the same genetic background and similar environmental conditions, different missense mutations can account for the severity of the disease.

Homozygous truncating mutations such as c.934delC, p.L312Ffs*30 and c.1513C>T, p.A505*, described by Janecke et al. have a severe phenotype with congenital onset of the disease (Janecke, Xu et al. 2017). Surprisingly, the homozygous frameshift mutation described by our group and Prasad

et al., c.7dup, p.S3Kfs*11, leads to a much less severe phenotype than the other truncating mutations and several missense mutations, such as S346I or R222W. This could be explained by a rescue ATG that is found in frame at nucleotide 22 (aminoacid 7) (http://atgpr.dbcls.jp/), possibly leading to a SGPL1 protein where only the first 6 aminoacids are missing at its N-terminus. SGPL1 does not have an identifiable signal peptide at the N-terminus; instead it has an anchor sequence at the transmembrane region. This could mean that the protein resulting from the rescue ATG could still be targeted to the ER and be partially functional, explaining the less severe phenotype found in these patients. Unfortunately, in all the cases, these frameshift mutations were not analyzed by western blot, nor were their enzymatic activity.

Also of interest are the heterozygous compound mutations that our group found in a family of French origin. The c.395A>G is predicted to create an exonic splicing enhancer sequence (http://ex-skip.img.cas.cz/) leading to the skipping of exon 5 and thus creating the frameshift allele p.I88Wfs*25, while the other allele carried the mutation c.832delA, p.R278Gfs*17. In patient fibroblasts we confirmed that there was skipping of exon 5 in all the transcripts analyzed by Sanger sequencing. Concordantly, no protein was observed by western blot, and the enzymatic activity in patient fibroblasts was barely detectable, even less than the S346I mutation. As such, we could anticipate that these heterozygous compound mutations lead to a very severe phenotype; however these patients show "only" SRNS onset in childhood, adrenal insufficiency diagnosed at one/two years of age, and mild and transitory ichthyosis in childhood, with no other organ manifestations. This can likely be explained by an exon skipping event that does not occur in 100% of the cases, and that may vary in different tissues or cell types. If exon 5 skipping does not occur, the resulting protein would have the missense mutation p.E132G, which we showed not to be deleterious to SGPL1 function in several functional assays including enzymatic activity of the overexpressed protein, yeast complementation experiments and fly rescue experiments.

Although the phenotypic variability can be mostly attributed to the mutation type itself, several findings point to a possible involvement of genetic modifiers or environmental factors that could alter the disease onset and progression. For example, in patients from three unrelated families that bear the same homozygous missense mutation R222Q, the onset of SRNS ranged between 2.5 years and 19 years and three patients still hadn't developed kidney disease at the age of 3 years and 8 years. Adrenal insufficiency was present in all the patients but age at diagnosis also ranged from the first months of life to 6.5 years. Moreover, peripheral neuropathy was only present in one of the 7 patients with the R222Q mutation.

It is quite possible that polymorphisms in other enzymes from the metabolic pathways of ceramides and S1P may be involved in modifying the onset of the disease and the affected organs. For instance, in *Drosophila*, decreased activity of SPHKs, leading to less S1P, or the presence of a hypomorphic allele of *lace*, the first enzyme of *de novo* ceramide biosynthesis, lead to a less severe *Sply* KO phenotype (Herr, Fyrst et al. 2003).

Environmental factors that directly inhibit SGPL1 function could also aggravate the disease; this is the case of THI (2-acetyl-4-(tetrahydroxybutyl)imidazole), a component of the caramel color III food additive, that has been shown to lead to inhibition of SGPL1 activity (Schwab, Pereira et al. 2005, Ohtoyo, Machinaga et al. 2016). Moreover, the availability of the SGPL1 cofactor pyridoxal phosphate, the active form of pyridoxine or B6 vitamin, could also influence the activity of the enzyme (Ohtoyo, Tamura et al. 2015). One could also imagine that specific types of lipids provided in the diet could also serve as modulators of the disease. For example, dietary supplementation with n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) has been shown to decrease sphinganine, S1P and lactosylceramide in the muscle of mice fed with a basal high fat diet, through an unknown mechanism (Kasbi-Chadli, Ferchaud-Roucher et al. 2016).

It could be interesting to determine exactly which metabolic enzymes are most important in modulating the disease phenotype. To identify loci involved in this type of modulation, the fly model could be extremely beneficial, enabling large scale second site modifier screens. The same kind of screening could be applied to see the impact of dietary factors on disease activity.

Mechanisms of the disease in different organs

SGPL1 loss of function and cellular pathogenic mechanisms

SGPL1 is a main regulator of sphingolipid availability through its terminal function on the single catabolic pathway of S1P. The pathogenic mechanisms leading to disease upon SGPL1 loss of function may be related to the accumulation of sphingoid species. This may directly lead to alteration of the physical properties of the cell membranes and lipid rafts, but also to altered intracellular and extracellular signaling due to a disrupted equilibrium between the bioactive lipids, ceramides and S1P. The fine balance between ceramides and S1P for instance has been showed to modulate several intracellular mechanisms, such as proliferation, apoptosis, intracellular calcium increase and autophagy. The decrease of SGPL1 end-products, phosphoethanolamine and hexadecenal, may also impair cellular homeostasis. In fact, phosphoethanolamine will ultimately be converted to

phosphatidylethanolamine, a major phospholipid of biological membranes, and hexadecenal will further be transformed into hexadecenoic acid and finally to palmitoyl-CoA and glycerophospholipids (Wakashima, Abe et al. 2014).

Interestingly the effects of SGPL1 depletion differ according to the cell type. While *Sgpl1*$^{-/-}$ MEFs proliferate well and are able to induce tumors when transferred to nude mice (Colie, Van Veldhoven et al. 2009), in neuron primary cultures from *Sgpl1*$^{-/-}$ mice or *in vivo* upon neural-targeted depletion of SGPL1, the inhibition of S1P degradation correlated with genotoxic stress and neuronal death (Hagen, Van Veldhoven et al. 2009, Hagen, Hans et al. 2011, van Echten-Deckert, Hagen-Euteneuer et al. 2014). Neuronal apoptosis induced by SGPL1 deficiency seems to be mediated by the accumulation of S1P generated by SPHK2, and by caspase-12 and calpain, activated by increases in intracellular calcium (Hagen, Van Veldhoven et al. 2009, Hagen, Hans et al. 2011). Interestingly the effect of S1P also seems to depend on the degree of cellular differentiation: while S1P stimulates proliferation of neural progenitor cells (Harada, Foley et al. 2004), it conversely induces apoptosis of terminally differentiated hippocampal neurons (Moore, Kampfl et al. 1999).

Accumulation of sphingoid bases and dyslipidemia

In affected individuals harboring *SGPL1* mutations, when serum sphingosine and S1P were measured, they were found to be increased (1.5 to 4 times (Atkinson, Nikodinovic Glumac et al. 2017, Janecke, Xu et al. 2017, Prasad, Hadjidemetriou et al. 2017)) and interestingly, ceramides of different carbon lengths showed even higher increases (in several cases more than 10 fold) (Prasad, Hadjidemetriou et al. 2017). Concordantly, in the Sgpl1 KO mice model, S1P, sphingosine and ceramides show a sharp increase in tissues and a significant increase albeit less in serum (Weber, Krueger et al. 2009) (Vogel, Donoviel et al. 2009). In the *Drosophila* model an increase in sphingosine and ceramides was observed in 3rd instar larvae lysates (our group and (Herr, Fyrst et al. 2003)).

In *SGPL1* mutated patients dyslipidemia as well as increased very long chain fatty acids were also observed (our group and (Prasad, Hadjidemetriou et al. 2017)). However it is difficult to distinguish if these are specific to SGPL1 deficiency or secondary to the changes in lipid profile induced by the NS.

Interestingly in *Drosophila* nephrocytes we observed a decrease in lipid droplets, which are known to be mainly composed of neutral lipids such as triacylglycerols. Also Prasad et al. described that in the adrenal cortex from Sgpl1$^{-/-}$ mice, cells from the corticosterone-producing zona fasciculate had fewer lipid droplets (Prasad, Hadjidemetriou et al. 2017). In fact, cholesterol and triacylglycerol levels can also be modulated by SGPL1, namely through the activation of sterol-regulatory element binding

protein (SREBP) (Dobrosotskaya, Seegmiller et al. 2002, Worgall 2011). However, what has been described is that SGPL1 deficiency leads to increased SREBP activation and cholesterol accumulation (Dobrosotskaya, Seegmiller et al. 2002, Vienken, Mabrouki et al. 2017), so other unknown mechanisms are possibly contributing to the observed phenotypes in nephrocytes and cells from the adrenal glands. In this regard, it is interesting to note that also in *Sgpl1*$^{-/-}$ mice there are tissue specific effects of lipid accumulation, as these accumulate in serum and liver parenchyma but these mice show almost no adipose tissue (Bektas, Allende et al. 2010). SREBP activation, lipid transporters and biosynthesis, as well as its degradation could be further investigated in order to clearly define the mechanism behind the decreased lipid droplets in nephrocytes.

Neuronal involvement

Atkinson et al. described an isolated axonal peripheral neuropathy in patients with *SGPL1* mutations (Atkinson, Nikodinovic Glumac et al. 2017). Our group also found peripheral neuropathy, sensorineural deafness, and central nervous system defects in some patients, namely corpus callosum defects and ataxia probably due to cerebellar involvement. Atkinson et al. performed experiments in the *Drosophila* model to define the pathogenic mechanism in neurons. Using the GAL4:UAS system, they specifically silenced Sply in neurons (using a pan-neuronal driver) and observed impaired morphology of the neuromuscular junction. Degeneration of chemo-sensory neurons was observed in adult flies, upon specific silencing in these cells (Atkinson, Nikodinovic Glumac et al. 2017). These results point to a cell-autonomous pathogenic mechanism of SGPL1 loss in neurons. Interestingly, it was recently shown that neural specific KO of *Sgpl1* in mice leads to cognitive deficits, accumulation of S1P and sphingosine in brain tissue, activation of the ubiquitin-proteasome system and impaired autophagy due to decreased phosphatidylethanolamine content, important for the formation of the phosphatidylethanolamine conjugate LC3-II that allows maturation of autophagosomal membranes (Mitroi, Deutschmann et al. 2016, Mitroi, Karunakaran et al. 2017). Intracellular S1P can also lead to genotoxic stress in neurons (Hagen, Hans et al. 2011), leading to a pro-apoptotic effect. Moreover, an imbalance in sphingolipids could also alter membrane rafts and its signaling. However, complex lipids such as sphingomyelin, one of the main components of myelin sheets, do not seem to be affected, as Atkinson et al. did not observe characteristics of a demyelinating neuropathy in *SGPL1* mutated patients.

Although intra-cellular mechanisms are for sure involved in the neural defects observed upon SGPL1 deficiency, extra-cellular S1P signaling, originating for example from glial cells, may also play a role. In mouse models, S1P extra-cellular signaling is active in the developing nervous system and both the

synthesis of S1P and expression of the S1PR1 are required for neurogenesis (Liu, Wada et al. 2000, Mizugishi, Yamashita et al. 2005). Moreover, in the adult nervous system, multiple S1PRs are expressed in neurons and glial cells (Chun and Hartung 2010).

Adrenal involvement

Our group, Prasad et al. and Janecke et al. (Prasad, Hadjidemetriou et al. 2017) (Janecke, Xu et al. 2017) have described adrenal dysfunction in patients carrying SGPL1 mutations. In all cases the adrenal insufficiency was found to be primary and not related to pituitary or hypothalamic defects and adrenal calcifications were a common radiological finding. In the patient cohort of Prasad et al. glucocorticoid deficiency was the most common finding, sometimes associated with mineralocorticoid deficiency. In agreement, expression of SGPL1 was higher in the cortical zone and lower in the medulla and capsule. The authors also showed that *Sgpl1* $^{-/-}$ mouse adrenal glands have defects in cortical zonation and an impairment of steroidogenesis (Prasad, Hadjidemetriou et al. 2017). In the literature, ceramides and sphingosine have been demonstrated to decrease steroidogenesis *in vitro* (Meroni, Pellizzari et al. 2000, Li, Ni et al. 2001), and sphingosine in particular binds the nuclear receptor steroidogenic factor-1, essential for steroid hormone-biosynthesis (Urs, Dammer et al. 2007). S1P on the other hand, has been shown to induce transcription of several steroidogenic factors *in vitro* (Lucki, Li et al. 2012). These data suggest that dysregulation of the ceramides and sphingosine:S1P equilibrium could be the main pathogenic factor in the adrenal gland.

Skin disease

Ichthyosis was described in the majority of the patients in our cohort and Prasad et al. (Prasad, Hadjidemetriou et al. 2017). Sphingolipids, and ceramides in particular, are very abundant in skin and essential for its barrier function (Uchida, Hara et al. 2000, Cha, He et al. 2016). SGPL1 deficiency could result in altered equilibrium of the ceramide species and by this mechanism lead to ichthyosis. Decreased hexadecenal production and subsequent impaired production of palmytoil-coA and glycerophospholipids could also play a role, as it is the case for mutations in *ALDH3A2*, which encodes a fatty aldehyde dehydrogenase that converts hexadecenal into hexadecenoic acid, and causes Sjogren-Larsson syndrome, a rare disease characterized by ichthyosis and intellectual deficit (Nakahara, Ohkuni et al. 2012).

Immune system defects

The defects in the immune system observed in SGPL1 mutated individuals are consistent with what has been reported in *Sgpl1* -/- mice. The mechanism of lymphopenia is secondary to the loss of the S1P gradient that provides the driving stimulus for lymphocyte egression from lymphoid tissues. It has been shown that specific deficiency of *Sgpl1* in dendritic cells is able to disrupt this gradient by increasing S1P tissue levels and thus perturb lymphocyte egress (Zamora-Pineda, Kumar et al. 2016). However, SGPL1 also plays a cell-autonomous role, as the specific *Sgpl1* KO in thymocytes leads to perturbation of T cell egress, although less severe than when targeting dendritic cells (Zamora-Pineda, Kumar et al. 2016). This could be mediated by increased S1P export and autocrine stimulation (Takabe, Paugh et al. 2008) and/or by reduced S1PR recycling to the plasma membrane (Gatfield, Monnier et al. 2014). Our group described a low number of CD4+ and CD8+ T lymphocytes and B lymphocytes in one of the SGPL1 mutated patients where a detailed immunological study was performed. Moreover expression of pro-inflammatory genes and cytokines was decreased after stimulation with LPS and phorbol myristate acetate/ionomycin, respectively. This however could be due to the low T cell count. Interestingly, the expression of TNFSF13B (BAFF) shows an extreme increase in response to phorbol myristate acetate/ionomycin stimulation. Increased expression of BAFF is linked to auto-immune disorders (Steri, Orru et al. 2017) and antibody mediated rejection of kidney grafts (Slavcev, Brozova et al. 2016). Although inflammatory infiltrates in tissues are not characteristic of SGPL1 deficiency, mice have been shown to have a pro-inflammatory profile of cytokines (Allende, Bektas et al. 2011). It would be interesting to check if other patients with *SGPL1* mutations also show increased expression of BAFF and to further study if this cytokine could play a role in the disease severity and manifestations.

Kidney disease

Most of the patients described here developed SRNS. Histologically the main lesion was FSGS although DMS was found in two cases of congenital NS. Podocyte foot process effacement was present and Prasad et al. described mesangial hypercellularity (Prasad, Hadjidemetriou et al. 2017). *In vitro* studies by our collaborators using a human podocyte cell line where SGPL1 was silenced using two different RNAis failed to show differences in the migration or proliferation of these cells. Moreover, when stimulated with S1P, WT podocytes did not show increased apoptosis. The same assays were performed in a rat mesangial cell line and the only positive finding was decreased migration that was shown to be mediated by S1PR1 and/or S1PR3. These results could point to a mesangial cell specific defect, with the podocyte being a bystander cell affected secondarily by an

unknown mechanism. Several criticisms can however be made to this assertion as 1) podocytes in culture do not represent the complexity of the podocyte *in vivo*, with their intricate 3D architecture, foot processes and slit diaphragms and continuous exposure to mechanical tension 2) the assays performed were quite limited and do not exclude that the podocytes could exhibit other kind of defects, such as accumulation of DNA damage, autophagy defects, defects in lipid rafts and consequently on the assembly of the slit diaphragm 3) the assessment of S1P-induced apoptosis was performed in WT podocytes and not in SGPL1 deficient podocytes, which may have been more susceptible. Furthermore, podocytes *in vivo* are terminally differentiated cells and are thus not able to complete mitosis, as such, even if we consider that S1P could have a pro-proliferative effect, this may prove to be deleterious *in vivo* 4) the level of SGPL1 silencing achieved by shRNA could be insufficient to induce a more overt phenotype, as low levels of residual enzyme can preclude manifestations of the disease (Vogel, Donoviel et al. 2009) 5) the decreased mesangial cell migration may have no effect on podocyte biology and it is difficult to interpret its value and consequences *in vivo*.

In the *Drosophila* model our findings point to a nephrocyte defect upon the ubiquitous loss of function of the SGPL1 ortholog, sply, as shown by the decreased density of foot processes and by the decreased albumin uptake. As already stated, nephrocytes also show a sharp decrease in the number of lipid droplets, suggesting disruption of the equilibrium between sphingolipids and other lipid species. As in *Drosophila* no S1PRs have been described, the effect that we observed on nephrocytes would argue in favor of a cell autonomous effect. This could be due to the possible deleterious effect of intracellular S1P or accumulation of intra-cellular ceramides, alteration of lipid raft signaling and consequently of the dockage of the slit diaphragm proteins and its signaling.

However, as we use a non-tissue specific sply loss of function model, a cell non-autonomous effect cannot be excluded, even if we consider that *Drosophila* do not have S1PRs. Increased extracellular S1P or ceramides in the hemolymph could be uptaken by endocytosis, and S1P could signal via a different type of receptor in *Drosophila*. Therefore, to distinguish between cell autonomous and non-autonomous effects, a specific silencing in nephrocytes should be performed. This could be done using the GAL4:UAS system in order to drive SGPL1 RNAis specifically in the nephrocyte using the nephrocyte specific promoter dot *vs* in the whole animal, using a ubiquitous promoter such as Tub. If a phenotype in nephrocytes was observed with both promoters, this would argue in favour of a cell autonomous effect. However, if the phenotype was only observed with the ubiquitous promoter, this would argue in favor of a cell non-autonomous effect.

It is tempting to think that both cell autonomous and non-autonomous mechanisms are leading to a phenotype in podocytes/nephrocytes. Besides the already mentioned intra-cellular pathogenic mechanisms, podocyte localization in the GFB could expose them to the higher concentrations of ceramides or S1P found in the serum of SGPL1 deficient patients. This could render them susceptible to apoptosis or genotoxic stress as it is the case in neurons (Hagen, Van Veldhoven et al. 2009, Hagen, Hans et al. 2011), terminally differentiated cells that have been compared to podocytes. Interestingly, extra-cellular S1P has also been shown to control the size selectivity of the blood brain barrier (Yanagida, Liu et al. 2017) and to modulate adherens junction assembly (Lee, Thangada et al. 1999), and a similar effect on the podocyte slit diaphragm is imaginable.

Further studies in mouse or *Drosophila* models with specific SGPL1 KO/KD in podocytes/nephrocytes and possibly exposure to increased serum/hemolymph S1P will be necessary to further address this question.

Potential therapies

As previously stated the reduction of ceramide synthesis by serine palmitoyl transferase or the decrease of S1P by the inhibition of sphingosine kinases have lead to an attenuated phenotype of SGPL1 loss of function in *Drosophila* (Herr, Fyrst et al. 2003). We could thus try to test drugs that reduce these sphingoid bases in order to modulate the disease severity. Moreover, supplementation of the catabolic endproducts of S1P, hexadecenal and phosphoethanolamine could also prove to be beneficial. In *Drosophila* for instance this could be feasible due to the existence of an easy read-out, which is the fly viability. We did attempt treatment with pharmacological inhibitors of SPHK1 and SPHK2 as well as medium supplementation with hexadecenal and phosphoethanolamine. However, with the conditions we used our results were negative. Factors that could have affected our results were the method and timing of drug administration (a single administration in the fly medium) and variability in egg laying number that indirectly affected the viability of the larvae due to competition for food/drug. Future experiments could be done using daily drug administration and by controlling more strictly for the number of individuals in each vial.

In the future, we could also envisage treatment with the recombinant SGPL1 enzyme, as it is done for other sphingolipidoses such as Fabry disease. However, addressing this protein to the ER, where it exerts its function, could be technically difficult to achieve. In light of the fact that the mutations are all loss of function, gene therapy may also present a potential therapeutic avenue, however effective targeting of the many affected organs may prove difficult. In this case, stem cell therapy could be the

solution. In fact, it has been shown that in cystinosis, a disease characterized by the deficiency of the lysosomal protein cystinosin, hematopoietic stem cells are able to infiltrate the affected organs and rescue the cellular defect through the transfer of cystinosin-bearing lysosomes to the cystinosin deficient cells via tunneling nanotubes that cross the basement membrane (Naphade, Sharma et al. 2015, Rocca, Kreymerman et al. 2015, Gaide Chevronnay, Janssens et al. 2016).

Part II

ADD3 and *KAT2B*, two novel SRNS candidate genes?

Article: A homozygous mutation in *KAT2B* extends the spectrum of *ADD3*-dependent intellectual disability syndrome.

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A homozygous mutation in *KAT2B* extends the spectrum of *ADD3*-dependent intellectual disability syndrome

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Short title: KAT2B is a candidate gene for SRNS and cardiomyopathy

Abstract

Recent evidence suggests that having more than one genetic diagnosis in a single patient is more common than previously anticipated. One of the challenges hereby is to dissect the contribution of each gene mutation, for which animal models such as *Drosophila* can provide a valuable aid. Here, we identified three families with mutations in *ADD3*, encoding for adducin-γ, with intellectual disability, microcephaly, cataracts and skeletal defects. In one of the families with additional cardiomyopathy and steroid-resistant nephrotic syndrome (SRNS), we found a homozygous mutation in *KAT2B*, encoding the lysine acetyltransferase 2B, suggesting that this second mutation might contribute to the increased disease spectrum. In order to define the contribution of *ADD3* and *KAT2B* mutation for the patient phenotype, we performed functional experiments in the *Drosophila* model as well as in cultured human cells. Confirming the pathogenicity of the *ADD3* and the *KAT2B* mutations, we found that both mutations were unable to fully rescue the viability of the respective null mutant of the *Drosophila* homologs, *hts* and *Gcn5*. While the *ADD3* mutation additionally impaired fly motor function, the *KAT2B/Gcn5* mutation showed a significantly reduced ability to rescue morphological and functional defects of cardiomyocytes and nephrocytes (podocyte-like cells). Interestingly, the simultaneous knockdown of *KAT2B* and *ADD3* synergistically impaired the adhesion and migration capacity of cultured human podocytes as well as kidney and heart function in flies, suggesting that mutations in both genes may be required for the full clinical manifestation. Altogether, our studies describe the expansion of the phenotypic spectrum in *ADD3* deficiency associated with a homozygous *KAT2B* mutation and thereby identify *KAT2B* as a novel candidate gene for SRNS and cardiomyopathy.

Author summary

Genetic diseases with complex syndromic constellations may be caused by mutations in more than one gene. Most examples studied so far describe genetic interactions of known disease genes, suggesting that a large number of multilocus diseases remain unexplored. Assessment of mutation pathogenicity can be achieved using animal models. One main advantage of using *Drosophila* is that it allows easy *in vivo* gene manipulation in cell types that are relevant for the disease. Here, we report the pathogenicity of *ADD3* mutations in three families with intellectual disability, microcephaly, cataracts and skeletal defects. Moreover, we provide evidence that the additional steroid-resistant nephrotic syndrome (SRNS) and cardiomyopathy in one of the families are a result of a missense mutation in the lysine acetyltransferase encoding *KAT2B* gene. In *Drosophila*, this mutation resulted not only in decreased viability, but also in functional defects in cardiomyocytes

and nephrocytes, which are cells with high similarity to mammalian podocytes. Our study implicates *KAT2B* as a candidate gene for SRNS and cardiomyopathy and emphasizes the importance of protein acetylation in kidney and heart function.

Introduction

The interrogation of the entire genome via next generation sequencing (NGS) technology has revolutionized clinical diagnostics. For medical genetics that traditionally focuses on finding monogenetic causes for Mendelian diseases, NGS has not only introduced much higher mutation detection rates but also unprecedented complexities. A recent retrospective analysis of more than 7000 exomes revealed multiple molecular diagnoses in around five percent of cases with suspected monogenic disease [1], suggesting that patients with multilocus diseases are underrecognized.

The phenotypic complexity of multilocus diseases, of which digenic disease represents the simplest and most common form, can be challenging for the physician, both when it comes to finding a diagnosis and to genetic counseling and risk assessment. Two distinct disease phenotypes in a single patient may present with a completely new clinical phenotype. On the other hand, two overlapping disease phenotypes may be misinterpreted as a single disease with increased severity.

The underlying genetic defects are equally difficult to predict. As a rule of thumb, compound phenotypes affecting different organ systems tend to be caused by mutations in two completely unrelated genes [1], whereas mutations in two genes encoding proteins from the same cellular pathway give rise to overlapping disease phenotypes [2-4]. But as genes can be pleiotropic, there are most likely many exceptions to this. Also, while one locus can be the primary locus [4], in other cases the two loci may be equal in importance [2].

One important challenge in modern human genetics is therefore to decompose the contributions of each gene mutation to the clinical phenotypes in question. So far, most reports on digenic inheritance in Mendelian disease have focused on known disease genes [1, 5, 6]. However, the diagnosis is even more difficult when dealing with genes that have previously not been associated with any genetic diseases. When this is the case, it is of utmost importance to perform functional validation in suitable experimental model systems.

In this study, we identify three families with mutations in *ADD3*, encoding for adducin-γ, with intellectual disability, microcephaly, cataracts and skeletal defects, establishing *ADD3* as a disease gene [7]. We further use mutation validation in *Drosophila* and mammalian cell culture to

demonstrate that in one of the families additional phenotypes in kidney and heart are associated with a homozygous missense mutation in the lysine acetyltransferase *KAT2B.*

Results

Clinical features of three families with intellectual disability and microcephaly

Six individuals in three families (families A-C) with intellectual disability and varying degrees of microcephaly (Table 1) were identified for this study. As additional clinical features, family A and B shared bilateral cataracts, corpus callosum defects as well as specific skeletal defects such as shortening of the third and fourth metatarsals (Fig 1A-D and Table 1), while the affected boy from family C suffered from epilepsy, severe speech delay and suspected cerebral palsy (Table 1). Moreover, the affected sibs in the consanguineous family A presented with steroid-resistant nephrotic syndrome (SRNS), a progressive renal disease characterized by podocyte lesions and massive proteinuria [8], and cardiomyopathy (Table 1). For individual II-1 and II-3, proteinuria was first detected at 7 and 12 years of age, respectively, and end-stage renal disease was diagnosed a decade later. Individual II-6 was diagnosed with SRNS and end-stage renal disease at the age of 13 years. In kidney biopsies, individual II-3 (Fig 1E) showed focal segmental glomerulosclerosis (FSGS) in a small percentage of glomeruli, while individual II-6 showed widespread FSGS lesions, hypertrophic and vacuolated podocytes (Fig 1E), associated with tubular atrophy, interstitial fibrosis and inflammatory cell infiltrates. In addition, all affected individuals from family A developed dilated cardiomyopathy with progressive heart failure and arrhythmia (Table 1). Cardiac failure was the cause of death for both individuals II-1 and II-3.

Whole-exome sequencing identifies missense mutations in ADD3 in all families

For the affected individuals in family A and C, the presence of mitochondrial disease was excluded by muscle biopsy. Moreover, high-resolution karyotypes were normal for all patients, and CGH arrays (performed for family B and C) did not show significant abnormalities. For further diagnostics, whole exome sequencing (WES) was thus performed on two affected members of family A as well as on the affected individuals and the parents of family B and C, after obtaining written informed consent and study approval. WES led to the identification of recessive potentially damaging mutations in *ADD3*, all segregating with the disease as confirmed by Sanger sequencing (*NM_016824.4:* family A: homozygous c.1975G>C, p.E659Q; family B: compound heterozygous c.86A>G, p.N29S c.1588G>A,

p.V530I (both on the same allele in the mother), c.995A>G, p.N332S (heterozygous in the father); family C: homozygous c.995A>G, p.N332S) (Fig 2A,B and Table 2). In 148,632 reference individuals from the gnomAD browser (http://gnomad.broadinstitute.org/), the ADD3 mutations were present at low frequencies and only in the heterozygous state (Table 2).

The identified *ADD3* mutations result in the substitution of amino acids located in the head and tail region of the protein product adducin-γ (Fig 2B). In humans, adducins form heterotetramers that are composed of either adducin-α and -γ (the most widely expressed) or adducin-α and -β (restricted mainly to erythrocytes and specific brain regions) [9]. These heterotetramers regulate the actin cytoskeleton by capping the barbed ends of F-actin and by promoting the interaction between actin and spectrin [9, 10]. Recently, a mutation in *ADD3* was shown to cause cerebral palsy, epilepsy, borderline microcephaly, thin corpus callosum and intellectual disability in one family [7]. As the phenotype of this family shows overlap with all our families, particularly family C, our study further supports the pathogenicity of the previously identified *ADD3* mutation.

A homozygous mutation in KAT2B associates with the extended phenotypes in family A

Family A, which was characterized by additional cardiomyopathy and SRNS, exhibited another potentially damaging homozygous mutation in lysine acetyltransferase 2B (*KAT2B).* The mutation (*NM_003884.4*: c.920T>C, p.F307S) segregated with the disease and was not present in the reference individuals from the gnomAD browser (Fig 2A and Table 2). *KAT2B* is known to acetylate a variety of substrates, including histones (preferentially H3), and to function as a transcription factor coactivator together with CBP/p300 [11-13]. The identified *KAT2B* missense mutation affects a highly conserved amino acid within the PCAF homology domain (Fig 2C), which is required for the interaction with CBP/p300 [14]. Thus, we reasoned that the *KAT2B* mutation, either alone or in synergy with the *ADD3* mutation, might be responsible for the renal and cardiac features observed in family A. To test this hypothesis, we used functional validation in cultured cells and *Drosophila melanogaster*.

ADD3 E569Q is a hypomorphic mutation in Drosophila

We first studied mRNA and protein expression in patient fibroblasts from affected members of family A by qPCR, western blotting and immunostainings. We found no significant decrease for ADD3 at the mRNA or protein level (Fig 3A and S1A,C Fig). By contrast, KAT2B protein levels were significantly

reduced despite unaltered mRNA levels (Fig 3B and S1B,D Fig), suggesting that the *KAT2B* mutation, but not the *ADD3* mutation, may have an impact on protein stability.

Next, we turned to *Drosophila* to assess the impact of the identified mutations as well as global gene disruption in the overall viability and specific organ function of an *in vivo*-model. *Drosophila hu li tai shao (hts)* corresponds to the sole homolog of all three adducin genes in humans. As previously described [15], hts^{null} hemizygous animals died at the late larval stage, with only a few escapers progressing into adult stage. The escapers showed rough eyes, uncoordinated movements and inability to fly leading to death within 24h after eclosion (S2A Fig). For mutation validation, we re-expressed in this hts^{null} background the human wild-type (WT) and mutant constructs using the ubiquitous driver *tubulin (tub)*-GAL4 (see S1 Table for precise genotypes). As E659, the amino acid mutated in adducin-γ, is located in a very poorly conserved region (Fig 2B), we performed rescue experiments with WT and mutated human adducin-γ. While re-expressing each of the adducins alone failed to rescue the viability, the co-expression of adducin-α and -γ (hereafter referred to as adducin-αγ WT) led to around sixty percent of viable mutant adults (Fig 3C). Accordingly, immunostainings showed that co-expression of adducin-α increases adducin-γ protein levels, suggesting that the stabilization of adducin-γ by adducin-α is a prerequisite for proper function (S3 Fig). Importantly, when co-expressing adducin-γ E659Q together with adducin-α (adducin-αγ E659Q), we observed a significantly reduced partial rescue of fly viability (Fig 3C). The surviving animals did not present with any defects in eye and wing morphology (S2A Fig) but showed climbing impairment in a geotaxis assay (S2B Fig) [7]. Altogether, these results suggest that *ADD3* E569Q is a hypomorphic mutation.

KAT2B F307S is a loss-of-function mutation in Drosophila

Drosophila Gcn5 is homologous with *KAT2B* and its paralog *KAT2A*. $Gcn5^{E333st}$ hemizygous animals died at late larval stage/early pupal stage as previously reported for this null mutation [16]. The expression of human *KAT2A* and *KAT2B,* either alone or in combination, with the ubiquitous *daughterless (da)*-GAL4 driver did not restore the viability of the mutant (Fig 3D). By contrast, the expression of *Drosophila Gcn5* with the same promoter led to a full rescue (hereafter referred to as Gcn5 WT; Fig 3D), suggesting that the human orthologs have evolved in structure and function in comparison to Gcn5. As the mutated amino acid in KAT2B, F307, is conserved in *Drosophila* Gcn5 (corresponding to Gcn5 F304), we re-expressed Gcn5 F304S in the $Gcn5^{E333st}$ hemizygous background (Gcn5 F304S). As a negative control, we re-expressed a predicted potentially damaging KAT2B variant (S502F corresponding to Gcn5 S478F) found in a homozygous state in a healthy individual from our

in-house database. While Gcn5 S478F rescue animals were normal (Fig 3D,E), Gcn5 F304S had a dramatically decreased viability with death occurring either in pupal stages or a few days after eclosion (Fig 3D). Adult escapers showed defects in wing and leg morphology, rough eyes and inability to fly (Fig 3E). Interestingly, this phenotype corresponds to what has previously been described for the deletion of the entire PCAF homology domain, where the mutation is localized [16]. We further detected acetylation defects for Gcn5 F304S but not for Gcn5 WT and control animals, as assessed by immunoblotting of larval nuclear extracts (Fig 3F). Altogether, the results suggest that KAT2B F307S is a loss-of-function mutation.

KAT2B F307S but not ADD3 E569Q causes renal defects in Drosophila

Since the presence of SRNS and heart defects in family A was the main phenotypic difference from the other families, we looked more specifically into the renal and cardiac system of the fly. The fly kidney is composed of garland and pericardial nephrocytes (Fig 4A) that perform the filtration of the hemolymph and Malpighian tubules that function as excretory tubes. The surface of nephrocytes is decorated with actin-anchored slit diaphragms showing high molecular similarity with those of mammalian podocytes [17-19]. Therefore, nephrocytes have successfully been used to functionally validate candidate genes for SRNS [20, 21]. By immunostaining, we observed that Hts localizes below the slit diaphragms at the cell cortex of nephrocytes (S4A,B Fig). Similarly, we found that adducin-γ is expressed in cultured human podocytes, where it also localized to the cell periphery (S5A,B Fig). In both cases, the localization pattern was specific as it was lost upon *hts* and *ADD3* knockdown (KD), respectively (S4B Fig and S5B Fig).

To study the requirements of Hts for the integrity of the slit diaphragm, we performed immunostainings for Kirre, the ortholog of the mammalian slit diaphragm protein Neph1 [22]. In line with the proposed role for adducin-γ in cortical actin regulation [23], we found that hts^{null} larval nephrocytes showed a decrease of Kirre between adjacent nephrocytes (S6A,B Fig). A similar observation was made for the KD of *hts* using *prospero (pros)*-GAL4 (S7A,B Fig).

Unlike the hts^{null} mutant, *hts* KD in nephrocytes did not compromise overall viability, allowing also the analysis of adult pericardial nephrocytes. We found that, while in 3-day-old adults *hts* KD did not affect pericardial nephrocyte number (S8A-C Fig), a significant decline of differentiated nephrocytes could be observed in 15-day-old adults (S8D-F Fig), consistent with previously reported characterizations of important podocyte genes [24-26]. Interestingly, in adducin-$\alpha\gamma$ rescue flies, no major difference with respect to Kirre localization in larval garland nephrocytes (S6A,B Fig) and the

number of adult pericardial nephrocytes (S9A,B Fig) was seen between the WT and the mutated form. Together, the results demonstrate that, while Hts is important for nephrocyte function, the *ADD3* missense mutation identified in family A does not cause a renal phenotype in flies.

For Gcn5, we found a prominent expression in nephrocyte and podocyte nuclei (S4C,D Fig and S5C,D Fig). $Gcn5^{E333st}$ hemizygous larvae presented with morphologically normal nephrocytes. However, we did detect a decreased H3K9 acetylation at this stage in the nephrocyte nuclei of the mutant, which could be rescued by Gcn5 WT but not by Gcn5 F304S (Fig 4B). At 15 days, the majority of adult Gcn5 F304S escapers showed mislocalized and/or abnormally shaped pericardial nephrocytes that were often reduced in number (Fig 4C-E). Similarly, the nephrocyte-specific expression of $Gcn5^{RNAi}$ caused a progressive decline of differentiated pericardial nephrocytes (Fig 4F,G and S8D-F Fig). Interestingly, unlike for the viability rescue, the expression of human KAT2B WT on the $Gcn5^{RNAi}$ background led to a partial rescue of nephrocyte number. However, this was not the case for KAT2B F307S (Fig 4F,G). These findings suggest that both Gcn5 F304S and KAT2B F307S impair the essential function of Gcn5 in nephrocytes.

KAT2B F307S but not ADD3 E569Q causes cardiac defects in Drosophila

The *Drosophila* heart is a tubular organ formed by contractile cardiomyocytes that pump the hemolymph (analogous to the blood in vertebrates) to the rest of the body. This organ system has proven to be an important tool for studying the genetics and pathophysiology of cardiac disease [27, 28]. Therefore, we studied heart function in adult adducin and Gcn5 rescue flies. As illustrated in the M-mode traces obtained from high-speed movies, adducin-$\alpha\gamma$ E659Q did not show any significant differences in heart period, cardiac output, fractional shortening and arrhythmia index when compared to adducin-$\alpha\gamma$ WT (S10A-H Fig). By contrast, Gcn5 F304S flies showed prolonged heart period and reduced cardiac output compared to Gcn5 WT and control flies (Fig 5A-E). Both Gcn5 WT and F304S rescue flies showed a reduction in the normal diastolic diameter compared to control flies (Fig 5F), but only for Gcn5 F304S there was a reduction in contractility, measured as fractional shortening (Fig 5G). Moreover, the Gcn5 F304S mutant showed a more irregular heartbeat compared to Gcn5 WT, reflected by an increase in the arrhythmia index (Fig 5H). In further support of Gcn5's requirement for normal heart function, the silencing of *Gcn5* with a heart-specific driver (*tin*>GAL4) led to a decreased cardiac output, an increased arrhythmia index and shortened diastolic diameter (S11B-D Fig). By contrast, the knockdown of *hts* did not cause any significant heart phenotypes (S11A-F Fig). This suggests that Gcn5 but not Hts is important for heart function in *Drosophila* and

that global overexpression of Gcn5 WT is more effective in restoring normal heart function in Gcn5-deficient flies than Gcn5 F304S.

The double knockdown of hts/ADD3 and Gcn5/KAT2B causes synergistic effects in Drosophila and human podocytes

Finally, to explore whether *ADD3* and *KAT2B* mutations could influence each other in the patients, we performed double KD in *Drosophila* cardiomyocytes and nephrocytes as well as human podocytes. In larval nephrocytes, the double KD of *Gcn5* and *hts* caused an increase in Kirre mislocalization, compared to the single KD (S7A,B Fig). Moreover, the double KD of *Gcn5* and *hts* caused a significant loss of differentiated nephrocytes already at 3 days post-eclosion (S8A-C Fig). We also tested whether the combined KD of *ADD3* and *KAT2B* might have an effect on podocyte adhesion and migration, which are processes typically affected by the silencing of SRNS genes [29, 30]. While *ADD3* and *KAT2B* double KD showed additive effects with respect to adhesion (S12A Fig), strong synergistic effects were found for the migration of podocytes (S12B Fig), suggesting that both in nephrocytes and podocytes the actin regulatory role of Hts/adducin-γ is supported by Gcn5/KAT2B function. Similarly, the cardiac-specific co-expression of hts^{RNAi} and $Gcn5^{RNAi}$ significantly aggravated the heart period length and the arrhythmia index observed upon single KD of *Gcn5* (S11A,C Fig), providing further evidence that the presence of mutations in both genes might be needed for overt disease manifestation in patients.

Discussion

Here, we identify *ADD3* mutations in three different families with similar neurological, skeletal and ophthalmological phenotypes, thereby confirming their pathogenicity. Moreover, we use functional validation in *Drosophila* and human cells to characterize the contribution of an additional mutation in *KAT2B* to the extended phenotype featuring one of the *ADD3* families. Our study provides an example of a multilocus disease due to a second mutation in a gene that lies on another chromosome and has hitherto not been associated with any genetic disease.

The severity of the *KAT2B* mutation in the fly model compared with the relatively late-onset cardiac and renal defects in the patients suggests a partial functional redundancy due to gene duplication in vertebrates. Accordingly, it has been shown that in mouse development loss of KAT2B can be compensated for by KAT2A [31]. Nevertheless, KAT2B is strongly expressed in mouse heart and

kidney, particularly podocytes [32, 33], while KAT2A has a more widespread expression pattern [14]. Moreover, adult mouse studies have shown that KAT2B can perform functions that are non-redundant with KAT2A [34-38], indicating that *KAT2B* deficiency alone could be sufficient for any clinical manifestations. What remains to be seen is whether the clinical impact of *KAT2B* deficiency needs to be unmasked by a sensitized genetic background, such as the *ADD3* mutation.

Vice versa, it is also possible that any renal and cardiac manifestation of the *ADD3* mutation requires additional gene variation(s). In this respect, it is interesting that rare but otherwise uncharacterized variants of *KAT2B* have been found to be enriched in a patient cohort with sporadic FSGS [32], suggesting that they contribute to disease susceptibility. Indeed, apart from histones, KAT2B has been shown to acetylate important cytoskeletal regulators in podocytes, encoded by genes mutated in FSGS and cardiomyopathy (e.g. actinin-4, TTC21B and myosin-7) [39]. Therefore, the combined use of our fly assay and diagnostic gene panels, including *KAT2B*, may in the future identify additional cases where *KAT2B* mutations are either primary causes or (epigenetic) modifiers of SRNS and cardiomyopathy.

Taken together, the identification of three families with *ADD3* mutations expands the mutational and phenotypic spectrum of *ADD3* deficiency reported initially. In addition, we provide evidence that the additional SRNS and cardiomyopathy in family A are a result of a missense mutation in *KAT2B*, most likely in combination with the *ADD3* mutation. Thus, our study proposes *KAT2B* as a candidate gene for renal and cardiac disease and highlights the importance of protein acetylation and epigenetics in kidney and heart function.

Materials and methods

Study participants

Following informed consent, we obtained clinical data, blood samples and skin biopsies from the affected individuals. Genomic DNA was isolated by standard procedures.The study protocol was approved by the Institutional review board - Comite de Protection des Personnes pour la Recherche Biomedicale Ile de France II – according to French law.

Whole exome sequencing

Whole-exome sequencing (WES) was performed for affected individuals II-3 and II-6 from family A

and for the two parents and the affected sib from family B and family C. Whole-exome capture was performed with the Agilent SureSelect Human All Exon Kit, 51Mb, V4 (family A), the Roche MedExome kit (family B) or a proprietary system from GeneDx (family C). The enriched library was then sequenced on either Life Technologies SOLID (paired end with 75+35 base pair (bp) reads; family A) or Illumina systems (family B: 2x150 bp reads; family C: 2x100bp read). Images were analyzed and the bases were determined according to Lifescope or bcl2fastq Conversion Software v2.17. Variants were called as described [40].

Fly strains and generation of transgenic flies

Crosses were maintained on standard cornmeal-yeast food at 25°C except for RNAi crosses (29°C). The fly stocks used in this study can be found in S1 Table. For *Hts*^{null} rescue constructs we used an N-terminal V5 tagged human *ADD3* (clone IMAGE: 6649991), WT or carrying the E659Q mutation, and the N-terminal HA tagged human *ADD1 (*gift from Vann Bennett, Duke University). For *Gcn5*^{null} rescue constructs we used the C-terminal HA tagged human *KAT2B* (clone IMAGE: 30333414), the N-terminal Flag tagged human *KAT2A* (gift from Laszlo Tora, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg) and the C-terminal Flag tagged *Drosophila Gcn5*, WT (gift from Clement Carré, University Pierre et Marie Curie) or carrying the mutations F304S or S478F (corresponding to human mutation F307S and S502F). All mutations were inserted using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Subsequently, the rescue constructs were subcloned into a *pUASTattB* vector (gift from *Konrad Basler*, University of Zurich) and injected into flies at *attP* landing sites by Bestgene, USA.

Cell culture

A conditionally immortalized human podocyte cell line developed by transfection with the temperature-sensitive mutant (tsA58) of the SV40-T-antigen-encoding gene, was kindly provided by Dr. Saleem (University of Bristol). In brief, the cells proliferated at the permissive temperature of 33 °C, whereas growth arrest and differentiation were induced by incubation at the nonpermissive temperature of 37°C for 14 days. Cells were grown with 7% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, insulin-transferrin-selenium, glutamine, penicillin and streptomycin (all from Life Technologies).

Primary skin fibroblasts were obtained from individual II-3 and II-6 from family A and two different age matched controls. These cells were grown in OPTIMEM medium supplemented with 20% fetal

bovine serum, glutamine, penicillin and streptomycin (all from Life Technologies) at 37°C with 7% CO2.

Establishment of Lentiviral Cell Lines

Small hairpin RNAs (shRNAs) Scramble (Scb) or targeting the 3'UTR of human *ADD3* and *KAT2B* mRNA in the lentiviral vector pLKO.1 were purchased from Sigma (*ADD3* clone: NM_019903.3-2280s1c1 TRCN0000123024; *KAT2B* clone: NM_003884.4-3192s21c1, TRCN0000364135). Lentiviral particles containing these constructs were produced in human embryonic kidney 293T cells as previously described [41]. *ShScb*, *ADD3* or *KAT2B* depleted podocytes were obtained by transduction with the respective shRNAs lentiviral particles and subsequent puromycin selection.

Human *ADD3 and KAT2B*, were subcloned from human full-length cDNA (*ADD3*: clone IMAGE: 6649991; *KAT2B* clone IMAGE: 30333414) into the expression vectors pLentiGIII and PLEX-MCS, respectively. An HA tag was added in frame, before the stop codon, to the C terminus of *ADD3* and *KAT2B*. The *ADD3* E659Q and *KAT2B* F307S mutations found in affected individuals were introduced with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All constructs were verified by sequencing. *ADD3* or *KAT2B* depleted podocytes were transduced with WT or mutant *ADD3* or *KAT2B* lentiviral particles, respectively.

RNA extraction, RT-PCR and Real Time quantification

Total RNA was isolated from fibroblasts using a Qiagen RNA extraction kit (Qiagen), following the manufacturer's instructions. cDNA was prepared using reverse transcriptase Superscript II (Invitrogen). PCR was performed using ReadyMix Taq PCR (Sigma). After RNA extraction and cDNA preparation by RT-PCR, relative expression levels of genes of interest were determined by real-time PCR using the Absolute SYBR Green ROX Mix (ABgene) and specific primers as follows: *ADD3* forward 5'- CTTGCTGGAATTGTTGTGGATAAG -3' and reverse 5'- CTGGTGGGCCATGATCATC-3'; *KAT2B* forward 5'- ATCACACGGCTCGTCTTTGAC -3' and reverse 5'- CACCAATAACACGGCCATCTT -3'. Experiments were repeated at least three times and gene expression levels were normalized to HPRT.

Immunoblotting

Total cell or third instar larvae total protein extractions were performed and the resolved proteins were probed using the primary antibodies: anti-PCAF rabbit monoclonal (3378, Cell Signaling,1:1000),

anti-adducin-γ mouse monoclonal (sc-74474, Santa Cruz, 1:1000) and anti-αtubulin mouse monoclonal (T5168, Sigma Aldrich, 1:5000). For immunoblotting of nuclear extracts [42], the primary antibodies anti-acH3K9 rabbit polyclonal (39918, Active motif, 1:1000) and anti-H3 mouse monoclonal (61475, Active motif, 1:1000) were used as well as the corresponding HRP-conjugated secondary antibodies (Amersham ECL, GE healthcare and Invitrogen). Bands were visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and quantified by densitometry using *Image J* software.

Immunofluorescence

Fibroblasts or podocytes were plated on noncoated coverslips or coverslips coated with rat-tail collagen type I (Corning), respectively. After 48h of culture cells were fixed with 100% ice-cold ethanol. Cells were incubated with a blocking solution (PBS, 1% BSA, and 0.1% tween 20) and further permeabilized for ten minutes with PBS 0.1% Triton. Incubation with the following primary antibodies was done ON at 4°C: anti-PCAF mouse monoclonal (sc-13124, Santa Cruz, 1:100), anti-adducinγ rabbit polyclonal (sc-25733, Santa Cruz, 1:100) and anti-HA (11 867 423 001, Roche, 1:200).

For immunofluorescence in *Drosophila,* garland and pericardial nephrocytes were dissected from third instar larvae and adults, respectively, and fixed for 20 minutes in 4% paraformaldehyde at room temperature and stained according to standard procedures. For Kirre stainings an alternative fixation method ("heat fixation") was used: nephrocytes were heat-fixed for 5 seconds at 90°C in 0.7% NaCl/0.05% TX-100 solution. The following primary antibodies were used: anti-Hts mouse monoclonal (#1B1 deposited to the Developmental Studies Hybridoma Bank (DSHB) by Lipshitz, H.D.), anti-Gcn5 rabbit polyclonal (gift from Jerry Workman, Stowers Institute for medical research, Kansas, 1:200) anti-Kirre rabbit polyclonal (gift from Karl Fischbach, Institute for Biology, Freiburg, Germany, 1:200), anti-Pyd2 mouse monoclonal (deposited to the DSHB by Fanning, A.S, 1:100), anti-acH3K9 rabbit polyclonal (#06-942, Upstate, 1:100), AlexaFluor488-conjugated anti-horseradish peroxidase (Jackson Immunoresearch, 1:400), anti-HA (11 867 423 001, Roche, 1:200) and anti-V5 rabbit polyclonal (v8137 Sigma, 1:200). The corresponding anti-isotype AlexaFluor antibodies (ThermoFisher Scientific, 1:200) were used at room temperature for 2 hours. Nuclei were stained with Hoechst. Confocal images were obtained with a Leica TCS-SP8 confocal microscope, and post-treatment analysis was performed with *Image J* software.

Statistical analyses

Results are presented as means ± standard error or standard deviation for the indicated number of experiments. Statistical analysis of continuous data was performed with two-tailed Student *t* test for pairwise comparisons or one-way analysis of variance for comparisons involving three or more groups, with Dunnet's, Bonferroni or Dunn post hoc test, as appropriate. Pearson's chi-squared test was used for analysis of categorical data. Linear relations between variables were analysed using linear regression analysis. $P<0.05$ was considered statistically significant. Analysis was carried out with GraphPad Prism software. (*$p<0.05$; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). All experiments were performed at least three times.

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Table 1. Clinical phenotype of affected individuals

	Family A			Family B		Family C	Kruer et al (3)
	II-1	**II-3**	**II-6**	**II-3**	**II-4**	**II-1**	**4 affected sibs (II-1, II-2, II-3, II-4)**
Sex	F	F	M	NK	F	M	II-1 and II-3: F II-2 and II-5: M
SRNS	Yes	Yes	Yes	NK	No	No	No
Age of onset of proteinuria (yrs)	7	12	<13	NA	No proteinuria	No proteinuria	NA
Renal histology	FSGS	FSGS	FSGS	NA	NA	NA	NA
Age of ESRD (yrs)	17	27	13	NA	NA	NA	NA
Heart disease	Dilated cardiomyopathy (dx 16 yrs), supra-ventricular arrhythmia (frequent auricular extra-systoles), heart failure	Dilated cardiomyopathy, arrythmia	Dilated cardiomyopathy (dx 8 yrs), arrhythmia (ventricular hyperexcitation), heart failure	NK	No	No	No
Neurological features	Borderline microcephaly Intellectual disability MRI – aspects of global demyelination Axonal demyelinating motor-sensory neuropathy	CP: -1SD Intellectual disability	Borderline microcephaly (CP: -2SD) Intellectual disability MRI – thin corpus callosum	Corpus callosum agenesis	Microcephaly (CP: -3SD), Intellectual disability - moderate MRI – partial agenesis of corpus callosum	Microcephaly (CP: -2.4 SD), Intellectual disability Intractable seizures MRI – possible cortical dysplasia	Borderline microcephaly (all sibs) Intellectual disability - mild to severe (all sibs) Spastic plegia (all sibs) Thin corpus callosum (II-2) Supranuclear gaze palsy (II-2) Epilepsy (II-2) Convergence-retraction nystagmus and strabismus (II-5) Strabismus (II-3)
Cataract	Congenital bilateral cataract	Congenital bilateral cataract	Bilateral cataract (6 yrs)	NK	Bilateral cataract	No	NK
Other features	Mild facial dysmorphy (wide nasal bridge) Arachnodactyly, lax joints, cubitus valgus, scoliosis Short stature	Dysmorphic features similar to the two brothers	Facial dysmorphy (wide nasal bridge, slight proptosis) Arachnodactyly, short 4^{th} and 5^{th} metatarsals, conical phalanges Lax joints, cubitus valgus scoliosis, spread iliac wings, short femural neck Microcytic anemia	NK	Mild facial dysmorphy (wide nasal bride, bulbous nasal tip, narrow palpebral fissures) Fifth finger mid-phalanx hypoplasia, short 4^{th} and 5^{th} metatarsals Short stature	Facial dysmorphy Short stature	
Age at last examination vs † age (yrs)	† 19	† 28	19	TOP	14	8	16 (II-1) 13 (II-2) 9(II-3) 3(II-5)

Abbreviations are as follows: CP cephalic perimeter; DD, developmental delay; ESRD, end-stage renal disease; F, female; FSGS, focal segmental glomerulosclerosis; yrs, years; M, male; MRI – magnetic resonance imaging NA, not applicable; NK, not known; SRNS, steroidresistant nephrotic syndrome; SD standard deviation; SS, short stature; TOP, termination of pregnancy; yrs, years; †, deceased

Table 2. Genetic variants identified in affected individuals with overlapping syndromes

Family/ Individual	Gene	Nucleotide change	Amino acid change	Zygosity, Segregation	MT	SIFT	PolyPhen-2	gnomAD allele frequencies
A/II-3, II-6	*ADD3*	c.1975G>C	p.E659Q	HOM	DC	0.13 (T)	0.980 (PD)	4/246110 (no HOM)
	KAT2B	c.920T>C	p.F307S	HOM	DC	0 (D)	0.990 (PD)	Not reported
B/II-3, II-4	*ADD3*	c.86A>G c.995A>G c.1588G>A	p.N29S p.N332S p.V530I	het m het p het m	DC DC DC	0.25 (T) 0.03 (D) 0 (D)	0.653 (PoD) 0.995 (PD) 1 (PD)	17/276960 (no HOM) 176/276966 (no HOM) 9/276822 (no HOM)
C/II-1	*ADD3*	c.995A>G	p.N332S	HOM	DC	0 (D)	0.995 (PD)	176/276966 (no HOM)

Abbreviations are as follows: D, deleterious; DC, disease causing; het, heterozygous; HOM, homozygous; m, maternal; MT, mutationtaster; p, paternal; PD, probably damaging; PoD, possibly damaging; T, tolerated;

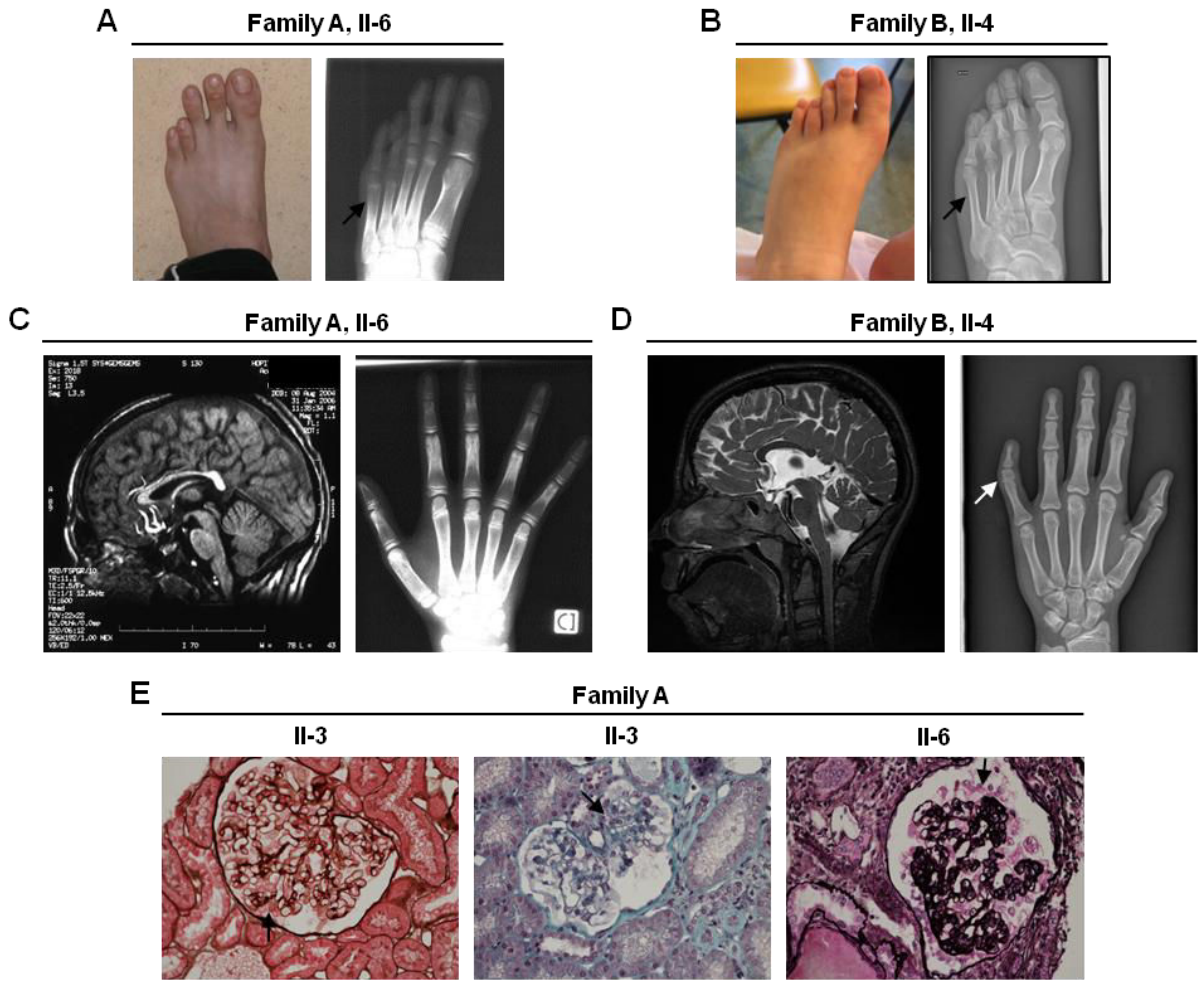


Fig 1. Clinical phenotypes of affected individuals

(A,B) Individuals II-6 (**A**; family A) and II-4 (**B**; family B) both show short 4th and 5th metatarsals.

(C) Individual II-6 (family A) shows thin corpus callosum in the brain MRI at 17 months as well as hand arachnodactyly in the skeletal radiography.

(D) Individual II-4 (family B) shows partial agenesis of the corpus callosum in the brain MRI at 14 years as well as mid phalanx hypoplasia in digit V (arrow) in the skeletal radiography.

(E) Kidney sections of affected individuals from family A. At 15 years old, 3 years after the proteinuria onset, individual II-3 showed mostly normal glomeruli but with hypertrophic podocytes (arrow) (left panel; methenamine-silver stain; 40x), while few glomeruli (middle panel) had segmental sclerosis of the glomerular tuft (arrow; trichrome stain; 40x). There were no major lesions of the tubular-interstitial compartment (left and middle). Individual II-6 (right panel) presented with end-stage renal disease at 13 years old and showed severe glomerulosclerosis of almost all the glomeruli with retraction of the glomerular tuft and hypertrophic podocytes (arrow) (methenamine-silver stain; 40x).

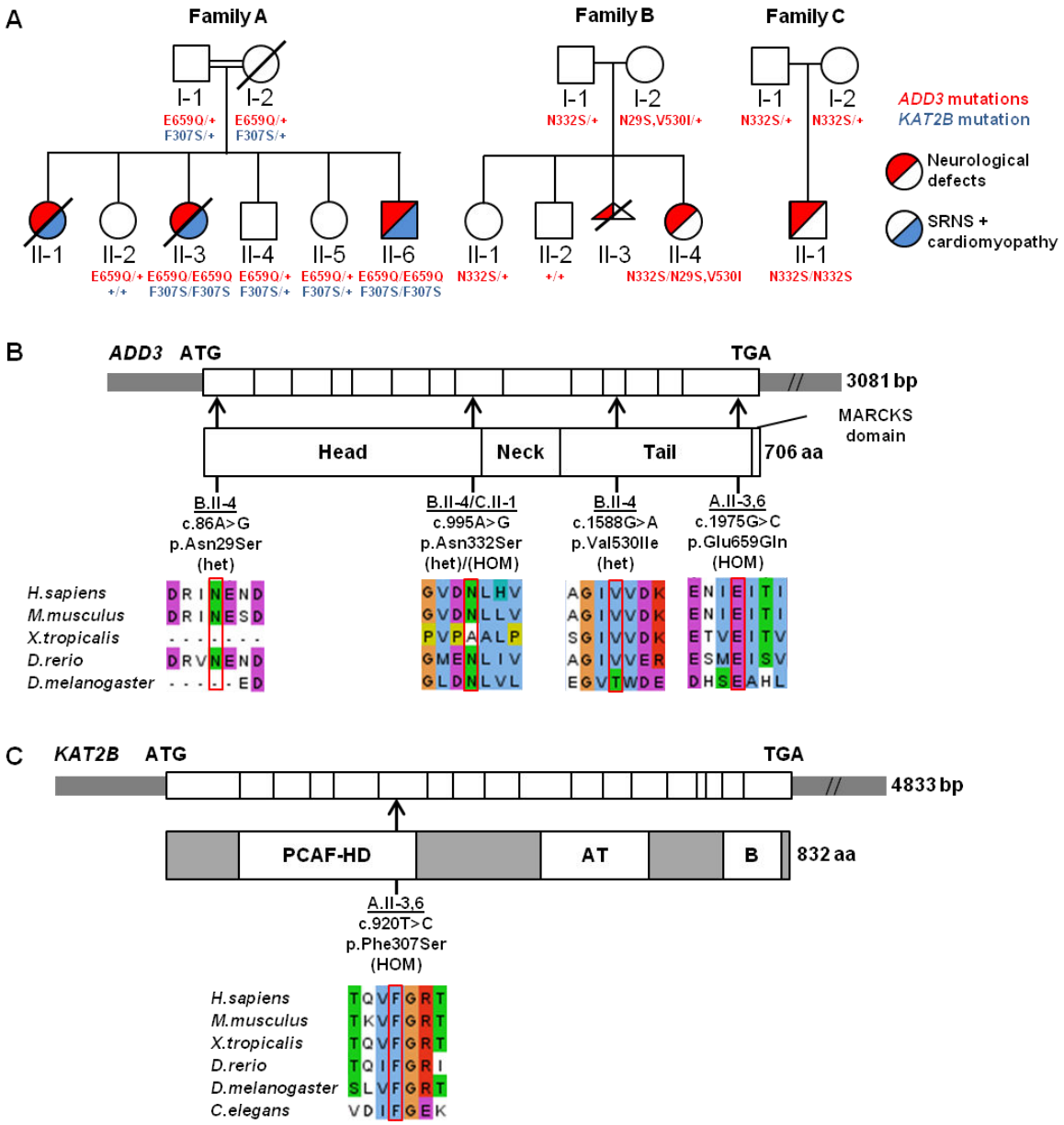


Fig 2. Identification of homozygous missense mutations in *ADD3* and *KAT2B*

(A) Pedigree and segregation status of mutations found in *ADD3* and *KAT2B* in family A. Discovery of *ADD3* mutations in family B and C was facilitated by GeneMatcher [43]. Half red coloured circles or squares denote patients with neurological defects and half blue coloured symbols denote patients with SRNS and cardiomyopathy. + symbols indicate non-mutated alleles. Mutations and segregation were confirmed by Sanger sequencing.

(B) Exon structure of human *ADD3* cDNA (long isoform NP_058432) and domains of adducin-γ protein. The relative position of *ADD3* mutations to protein domains and exons are indicated (arrows). All mutations also affect the short isoform of *ADD3* (NP_001112). Below each mutation, the phylogenetic conservation of the altered amino acid residues is shown.

(C) Exon structure of human *KAT2B* cDNA and domains of KAT2B protein. PCAF-HD, p300/CBP-associated factor homology domain; AT, acetyl transferase domain; B, Bromo domain. The relative position of *KAT2B* mutation to protein domains and exons are indicated (arrow). The phylogenetic conservation of the altered amino acid residue is shown.

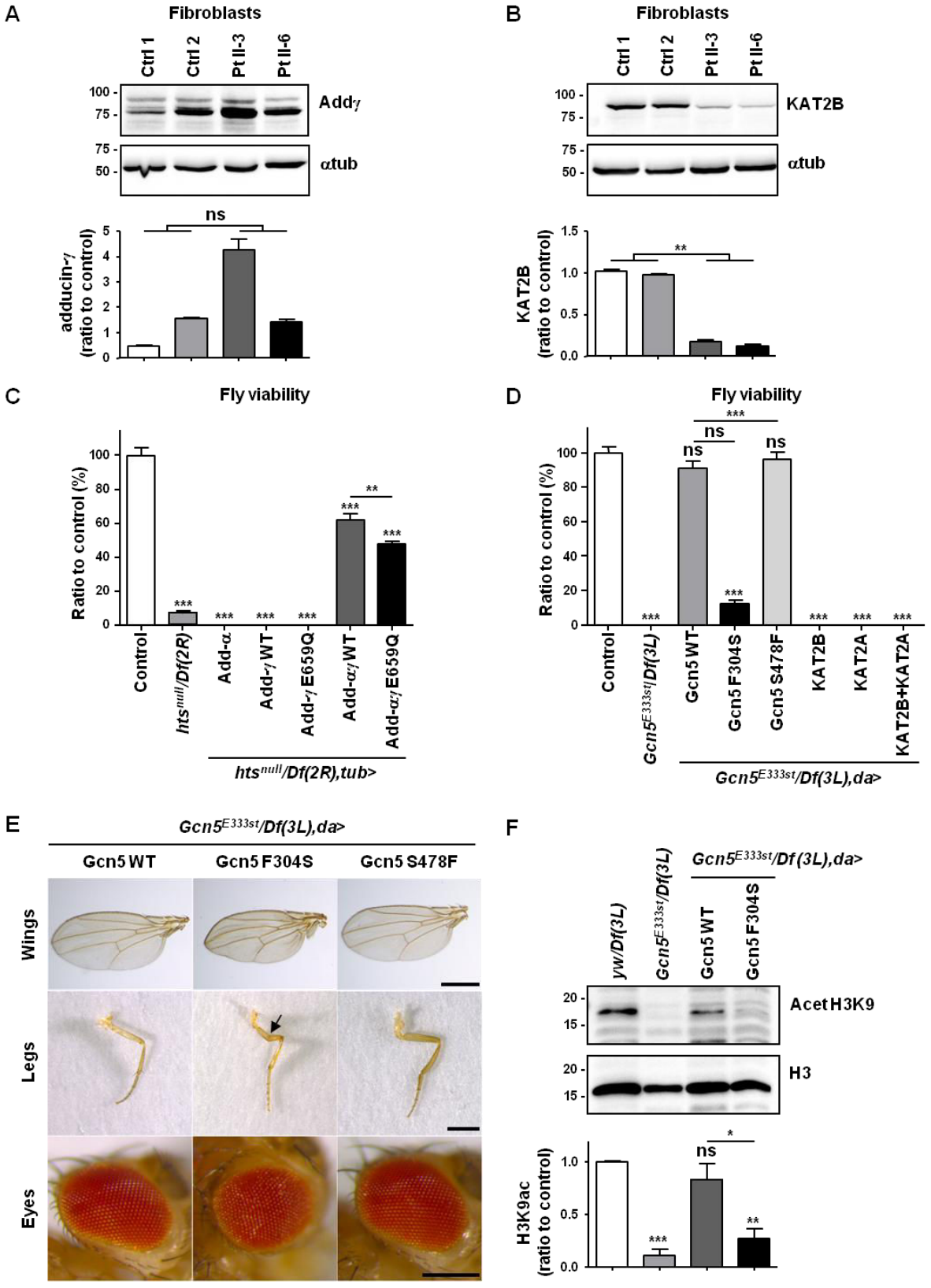


Fig 3. Effect of *ADD3* and *KAT2B* mutations on protein levels in fibroblasts and on viability and morphology in *Drosophila*

Fig 3. Effect of *ADD3* and *KAT2B* mutations on protein levels in fibroblasts and on viability and morphology in *Drosophila*

(A,B) Adducin-γ **(A)** and KAT2B **(B)** protein levels in control and patient fibroblasts. Lysates of patient II-3 and II-6 fibroblasts and age-matched control fibroblasts (Ctrl 1 and 2) were analyzed by western blotting. Results were normalized to the loading control α-tubulin. Each quantification is shown in the lower panel (n=3 independent experiments, student's t-test).

(C) Viability for hts^{null} hemizygous flies and respective rescues with adducin (Add) construct(s) using *tubulin*-GAL4 (*tub>*). After 48h of egg laying on standard cornmeal/yeast food, viability was calculated as the percentage of hatching adults of the indicated genotype and normalized to the control. The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between *Df(2R)BSC26* (harbouring the *hts* gene) and a non-overlapping deficiency on the same chromosome (*Df(2R)247*). Quantification is for >100 F1 eclosing flies/genotype/experiment in 5 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test.

(D) Viability for $Gcn5^{null}$ hemizygous flies and respective rescues with Gcn5 and KAT2A/B construct(s) using *daughterless*-GAL4 (*da>*). Viability was assessed as described in **(B)**. Human *KAT2B* F307S and S502F mutations correspond to *Gcn5* F304S and S478F mutations, respectively. Gcn5 S502F variant predicted to be deleterious (PolyPhen-2 score of 0.98) was found on a healthy individual at the homozygous state in our in-house exome database. The control corresponds to the viable F1 trans-heterozygous flies obtained from the cross between *Df(3L)sex204* (harbouring the *Gcn5* gene) and a non-overlapping lethal mutant on the same chromosome ($CG3103^{0MI0010}$). Quantification is for >100 F1 eclosing flies/genotype/experiment in 5 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test.

(E) Phenotype of Gcn5 WT, Gcn5 F304S and Gcn5 S478F rescue animals. Pictures correspond to adult flies one day post-eclosion and are representative of the defects found in wings (separated wing blades), legs (femur kinking, arrow) and eye (small and mild rough eye). Scale bars: wings: 500µm, legs: 500 µm, eye: 200µm.

(F) H3K9 acetylation levels of $Gcn5^{null}$ and Gcn5 WT and F304S rescue animals. Extracted nuclear proteins from 3rd instar (=late) larvae were analysed by western blotting normalized to non-acetylated Histone 3 (H3). Quantification is shown in the lower panel (n=3 independent experiments; one-way ANOVA with Bonferroni post-test).

For all panels: ns, non significant, *p<0.05 **p<0.01, ***p<0.001 (see S1 Table for details on transgenic flies).

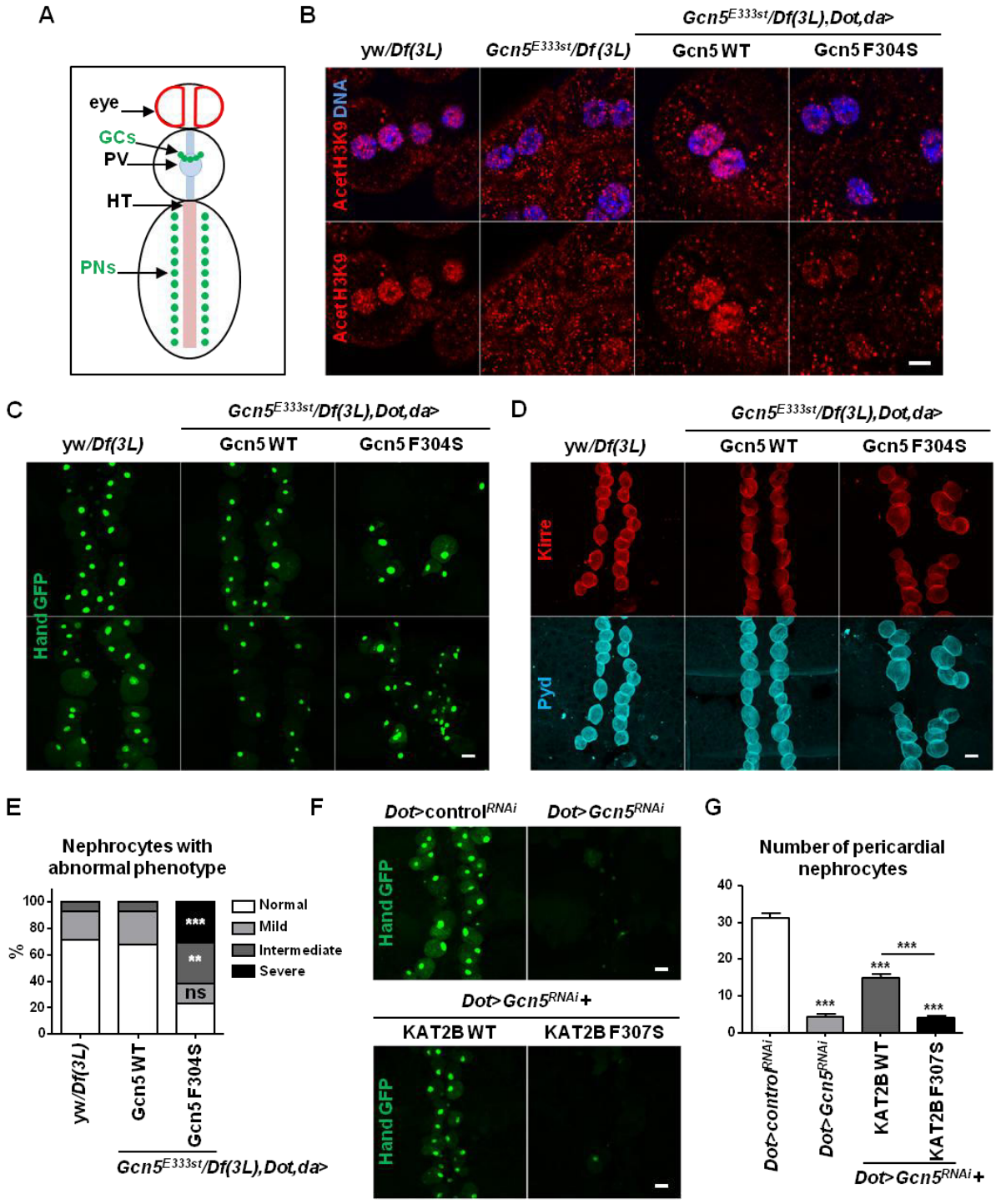


Fig 4. Effect of *Gcn5/KAT2B* mutation on histone acetylation and survival of *Drosophila* nephrocytes

(A) Schematic drawing of the localization of garland nephrocytes (GCs) and pericardial nephrocytes (PNs). The garland cells are attached to the proventriculus (PV) whereas the pericardial nephrocytes are lining the heart tube (HT).

(B) Acetylated H3K9 in larval garland nephrocytes of *Gcn5*^{null} and Gcn5 WT and mutant rescue animals. *Dorothy (Dot)*-GAL4 (a nephrocyte specific promoter) is used in combination with *da*-GAL4

as the latter shows only minor expression in nephrocytes. Garland nephrocytes of the indicated genotypes were stained for acetylated H3K9 (red) and Hoechst (blue). At the time of dissection, larvae were in the third instar stage (the same for all other garland nephrocyte stainings). Scale bar: 5 µm.

(C) Pericardial nephrocytes in adult *Gcn5* rescue mutant flies (7-15 days after eclosion) that express transgenic GFP (green) driven under the *Hand* promoter (*Hand*-GFP), specific for nephrocytes and cardiomyoblasts. Dissected pericardial nephrocytes were fixed with PFA and observed directly for GFP signal. Scale bar: 30 µm.

(D) Pericardial nephrocytes in adult *Gcn5* rescue mutants (7-15 days after eclosion). Dissected pericardial nephrocytes of the indicated genotypes were stained for the differentiation markers Kirre (red) and Pyd, corresponding to ZO-1 in vertebrates (blue). Scale bar: 30 µm.

(E) Quantification of the pericardial nephrocyte defects found in $Gcn5^{null}$ rescue mutants (n>13/genotype; 3 independent experiments; Chi-square test). Nephrocytes with abnormal phenotypes included nephrocytes with abnormal distribution, abnormal shape, multinucleated or fragmented nuclei and reduced number of nephrocytes (<20). Phenotype severity was scored as normal (0), medium (1), intermediate (2) and severe (>2).

(F) *Dot-GAL4*-mediated *Gcn5* KD in pericardial nephrocytes from 15 days old flies on the *Hand*-GFP transgenic background with co-expression of KAT2B WT or KAT2B F307S. $TRPP2^{RNAi}$ was used as a control RNAi, as *TRPP2* is a testis-specific gene [44]. Scale bars: 30 µm.

(G) Quantification of the number of nephrocytes as GFP positive cells surrounding the heart (n>20/genotype; 3 independent experiments, Kruskal-Wallis with Dunn's post-test).

For all panels: ns, non significant, **p<0.01, ***p<0.001 (see S1 Table for details on transgenic flies).

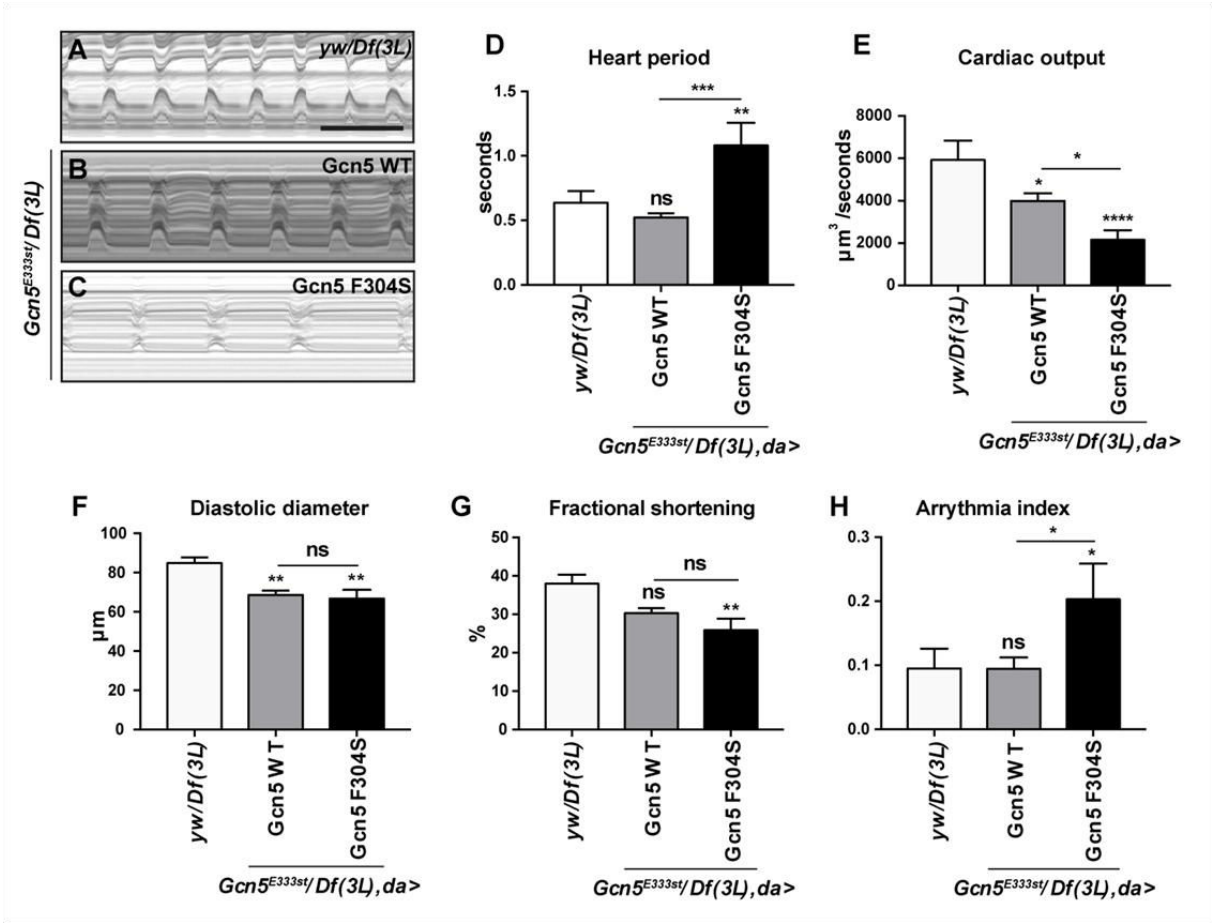
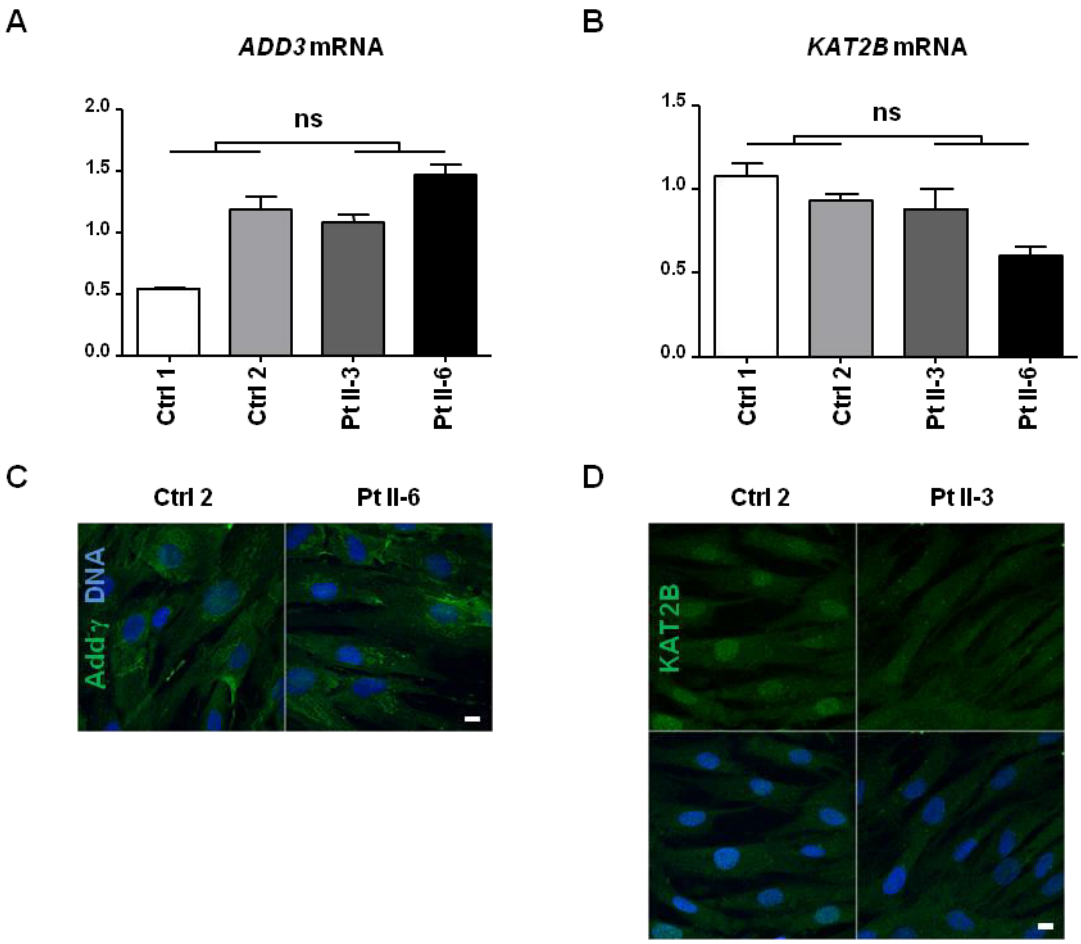


Fig 5. **Effect of Gcn5 F304S mutation on *Drosophila* heart function**

(A-C) M-mode kymographs of 1 day old beating hearts of control flies (*yw/Df(3L)*; A) and Gcn5^{null} flies rescued with Gcn5 WT **(B)** or Gcn5 F304S **(C)**. Scale bar: 1 second.

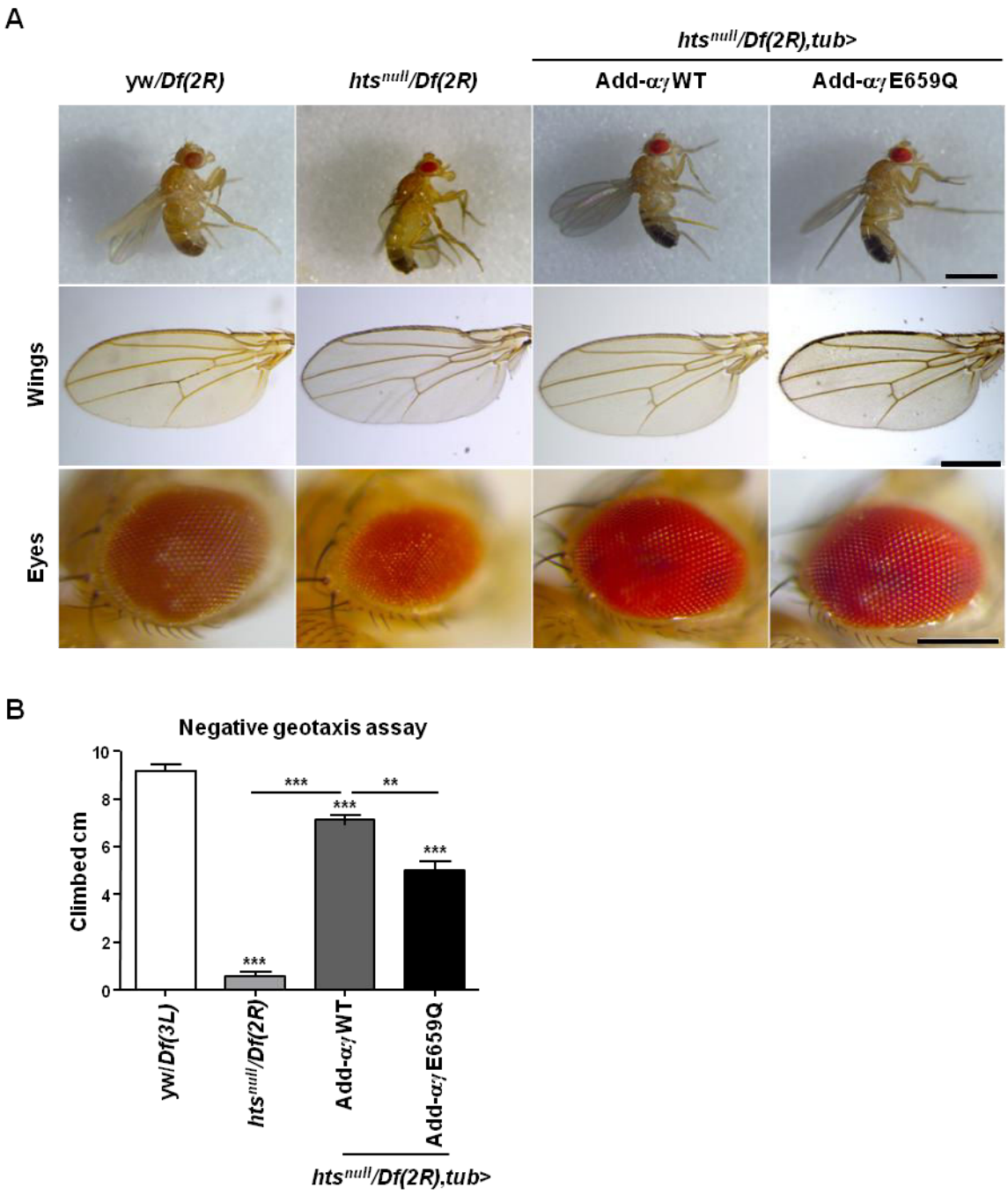
(D-H) High-speed movies of beating hearts were analysed using semi-automated Optical Heartbeat Analysis [45]. For quantification, 8-19 flies were analyzed. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison for all parameters except arrhythmia index **(H)**, which was analysed using Mann-Whitney-Wilcoxon.

For all panels: ns, non significant, *p<0.05 **p<0.01, ***p<0.001, ****p<0.0001 (see S1 Table for details on transgenic flies).



S1 Fig. mRNA levels and subcellular localization of ADD3 and KAT2B in control and patient fibroblasts

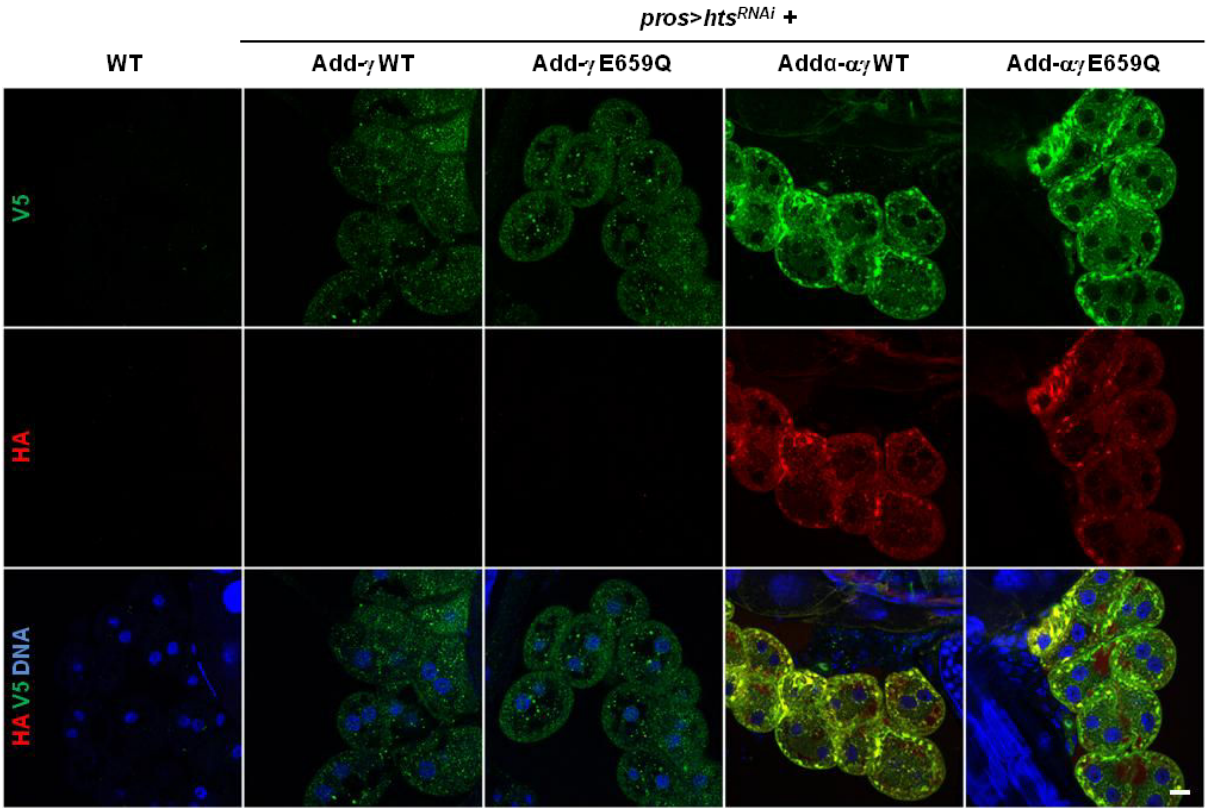
(A,B) *ADD3* (A) and *KAT2B* (B) mRNA levels in patient fibroblasts were assessed by quantitative PCR. Experiments were repeated at least three times and gene expression levels were normalized to the house keeping gene *HPRT*. Statistical analysis was performed using student's t-test; ns, non-significant. **(C,D)** Immunostaining was performed for adducin-γ (green; C) and KAT2B (green; D) in control and patient fibroblasts. Nuclei were stained with Hoechst (blue). Note the loss of nuclear staining for KAT2B in patient fibroblasts. Scale bars: 10 µm.



S2 Fig. Morphology of *hts*^{null} flies and adducin-αγ WT or E659Q rescue mutants

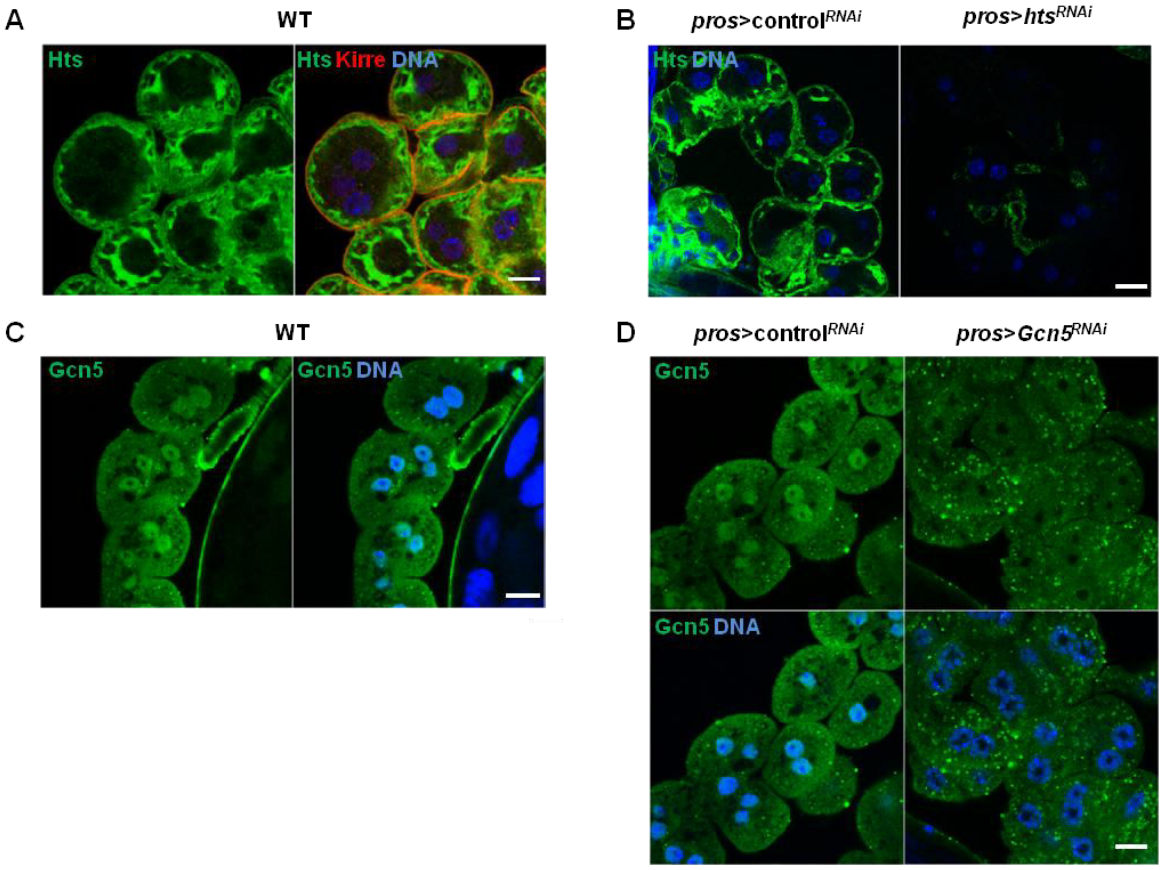
(A) Representative pictures of *hts*^{null} and respective adducin-αγ WT and E659Q rescue mutants one day post-eclosion. *hts*^{null} flies have rough eye and motor coordination defects and are unable to fly. The ubiquitous co-expression of adducin-α and -γ using *tub*-GAL4 rescues these defects regardless of the presence of the E659Q mutation. Scale bars: upper panel: 1mm, wings: 500µm, eye: 200µm. **(B)** Negative geotaxis assay for one-day-old adult flies. Flies were transferred to a graduated tube, and after tapping, the length climbed in 8 sec was recorded [46]. Quantification was performed on 6

independent experiments with >38 flies/genotype using one-way ANOVA with Kruskal-Wallis post-test. ns, non significant (see S1 Table for details on transgenic flies).



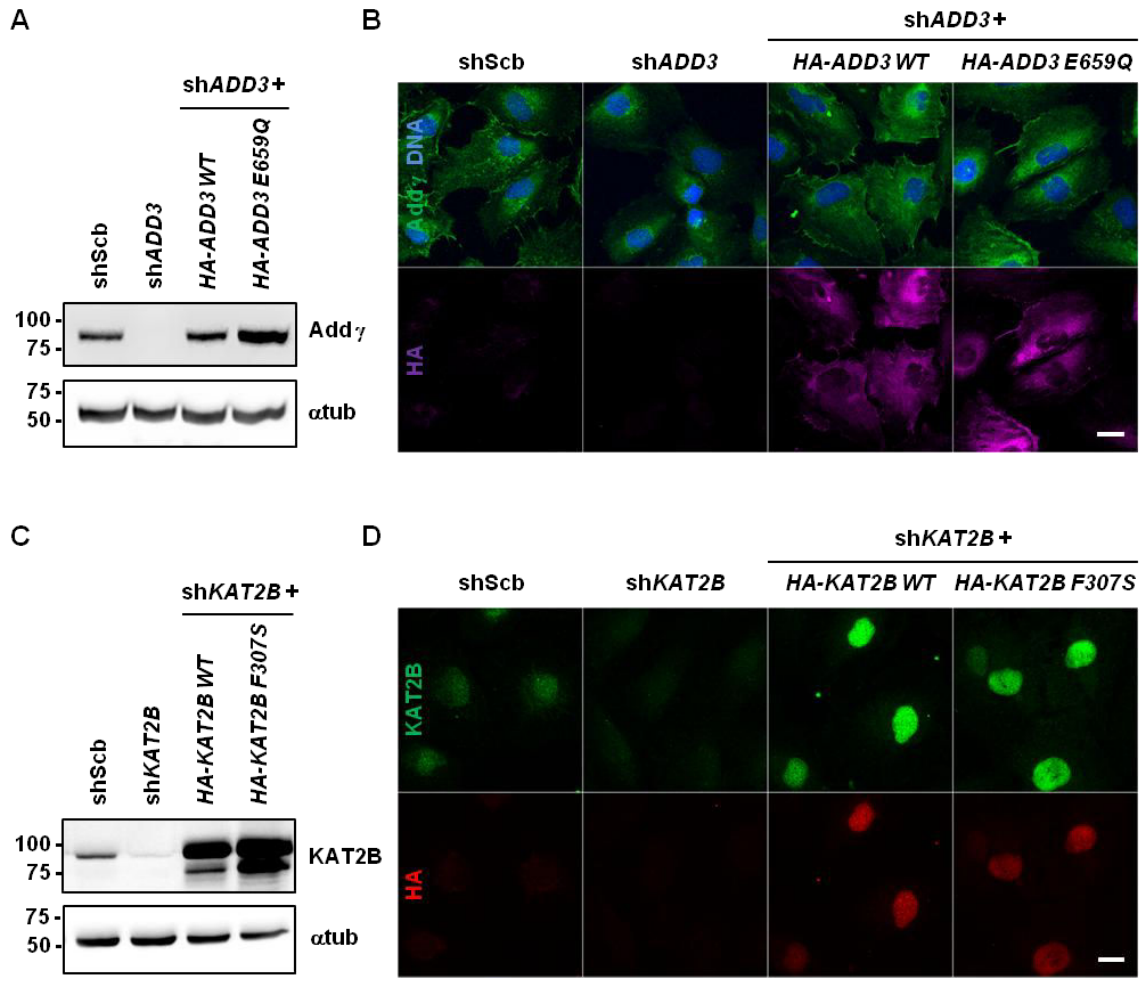
S3 Fig. Adducin-α and -γ co-expression in garland nephrocytes

The KD of *hts* and simultaneous re-expression of human HA-tagged adducin-α and V5-tagged adducin-γ was performed in garland nephrocytes with *prospero (pros)-GAL4* (see S1 Table for details on transgenic flies). Dissected garland nephrocytes of the indicated genotypes were stained for HA (green) and V5 (red). Nuclei were stained with Hoechst (blue). Scale bar: 10 µm.



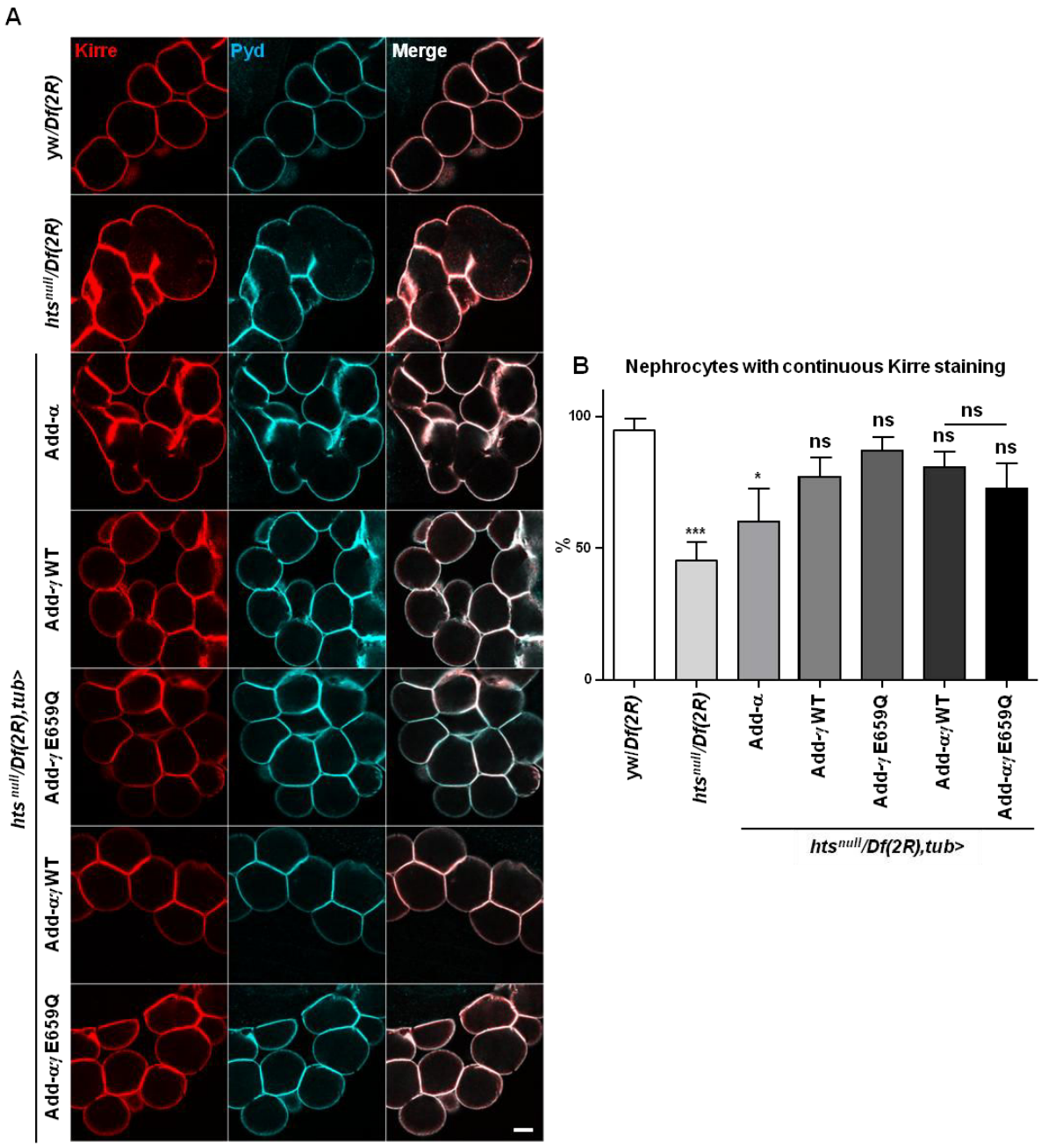
S4 Fig. Expression and localization of Hts and Gcn5 in garland nephrocytes

(A-D) Dissected wild-type (WT) garland nephrocytes were stained for Hts (A) and Gcn5 (C). Kirre is in red (A). Nephrocyte-specific KD of *hts* (B) and *Gcn5* (D) in nephrocytes using *pros*-GAL4. Dissected garland nephrocytes of the indicated genotypes (see also S1 Table) were stained for Hts (green; B) and Gcn5 (green; D). Nuclei were stained with Hoechst (blue). Scale bars: 10 µm.



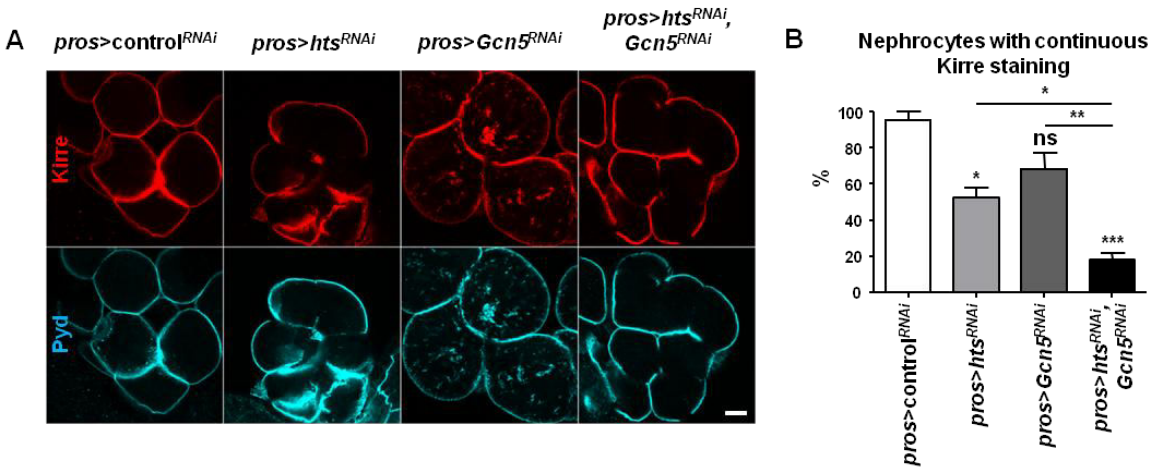
S5 Fig. Expression and subcellular localization of adducin-γ and KAT2B in podocytes

(A) Cell lysates from undifferentiated podocytes were analysed by western blotting using anti-adducin-γ. Anti-α-tubulin was used as a loading control. **(B)** Differentiated podocytes were stained for adducin-γ (green), HA (magenta) and DNA (blue). **(C)** Undifferentiated podocyte cell lysates were analysed by western blotting using the anti-KAT2B. Anti-α-tubulin was used as a loading control. **(D)** Differentiated podocytes were stained for KAT2B (green) and HA (red). Scale bars: 20 μm.



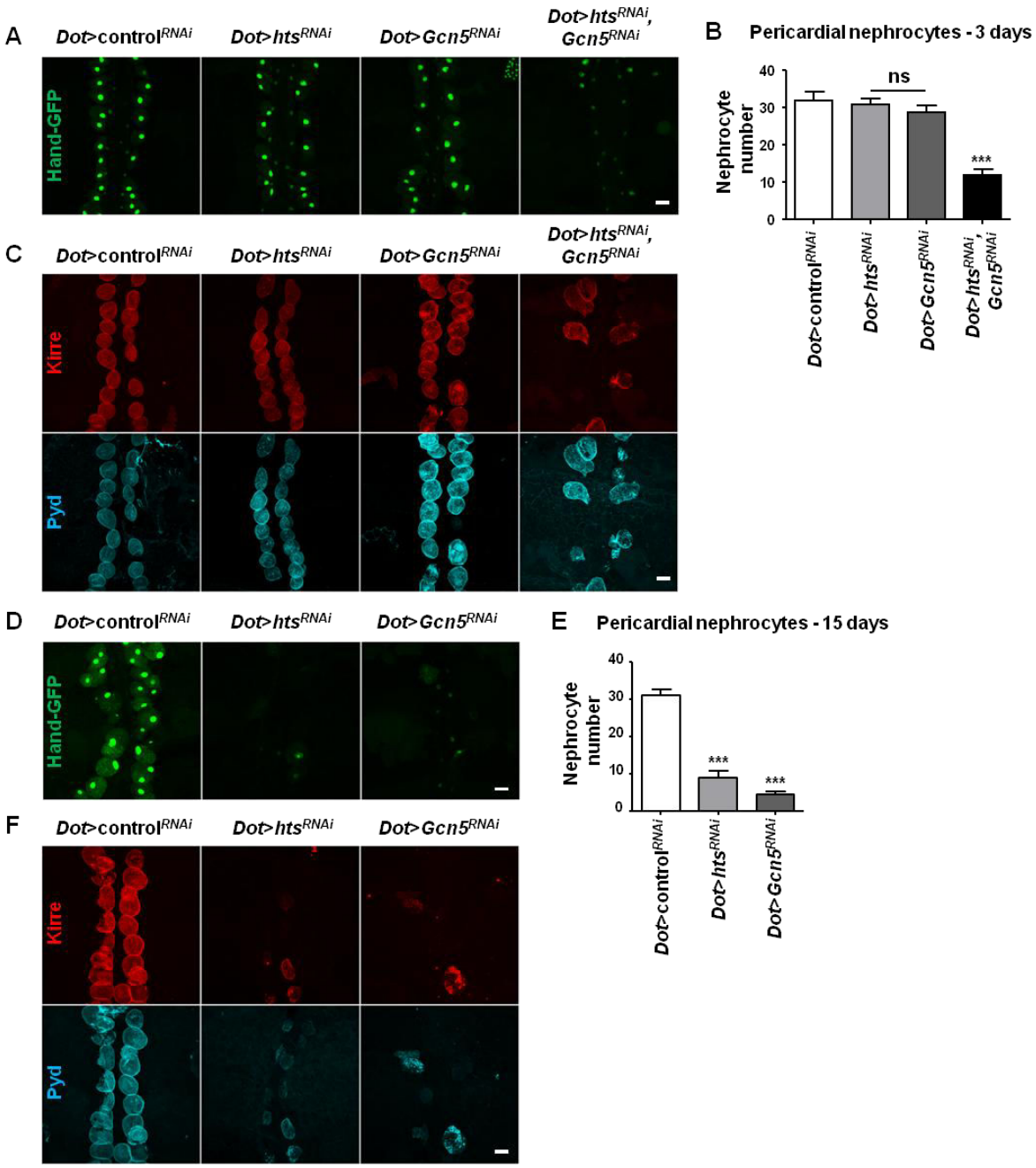
S6 Fig. Garland nephrocyte phenotype of *hts*^{null} and adducin-αγ rescue mutants

(A) Kirre and Pyd localization in *hts*^{null} and rescue mutant nephrocytes. Dissected garland nephrocytes of the indicated genotypes were stained for Kirre (red) and Pyd (blue). Scale bar: 10µm. **(B)** Quantification of nephrocytes showing a continuous Kirre staining using >9 samples/genotype from 3 independent experiments Statistical analysis was performed with Kruskal-Wallis with Dunn's post-test. ns, non significant, *p<0.05, ***p<0.001 (see S1 Table for details on transgenic flies).



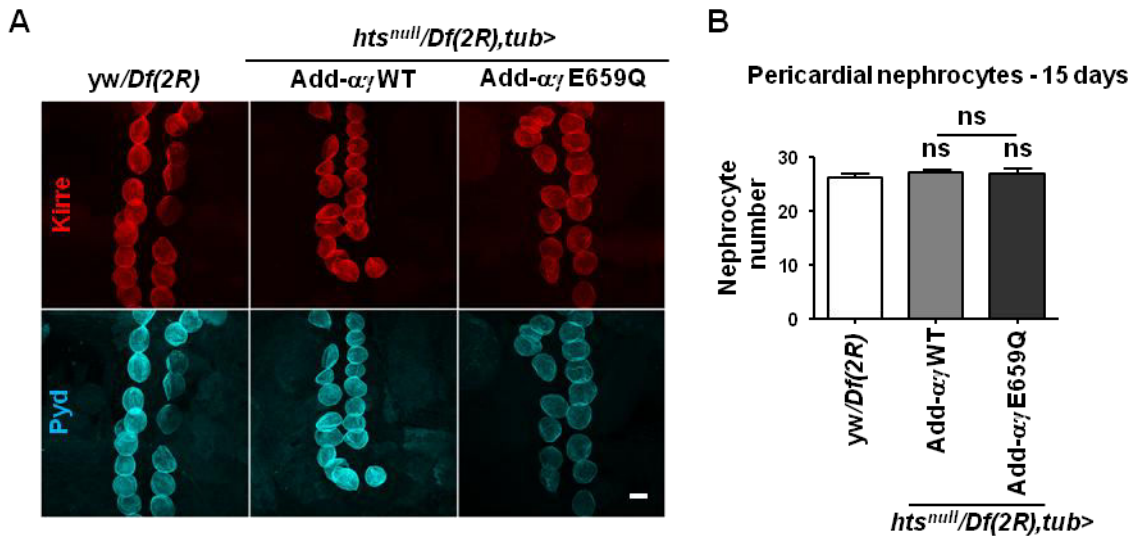
S7 Fig. Kirre localization in nephrocytes in single and double KD of *hts* and Gcn5

(A) *Pros-GAL4*-mediated KD of *hts* and/or *Gcn5* in garland nephrocytes. Dissected garland nephrocytes of the indicated genotypes were stained for Kirre (red) and Pyd (blue). Scale bar: 10µm. **(B)** Quantification of nephrocytes showing a continuous Kirre staining using >12 samples/genotype in 3 independent experiments. Statistical analysis was performed with Kruskal Wallis with Dunn's post-test.



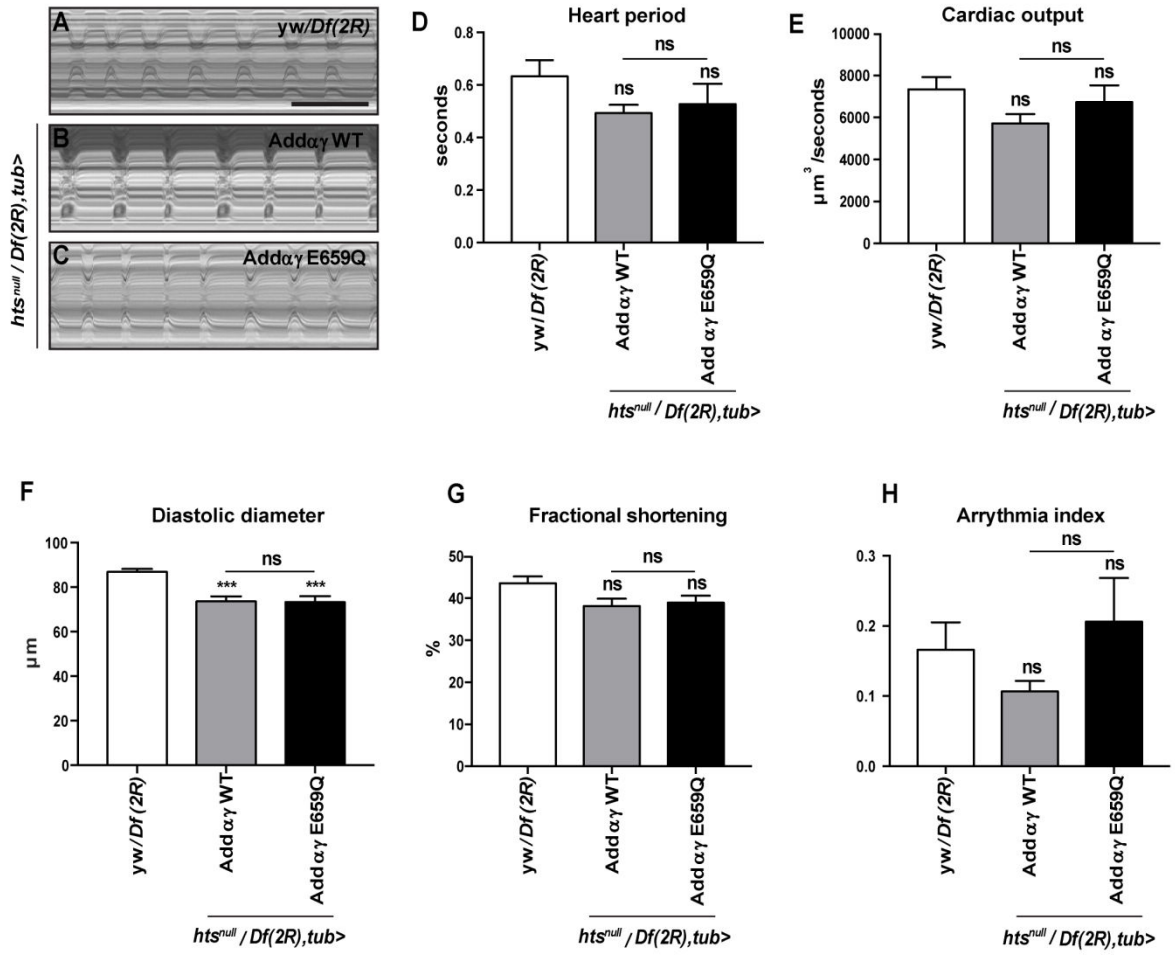
S8 Fig. Number of pericardial nephrocytes in single and double KD of *hts* and *Gcn5*

(A-F) *Dot-GAL4*-mediated KD of *hts* or *Gcn5* in pericardial nephrocytes. Pericardial nephrocytes of adult flies with the Hand-GFP background were observed directly for GFP signal after fixation (A,D). Immunostaining was performed for the differentiation markers Kirre (red) and Pyd (blue; C,F). Images are representative of pericardial nephrocytes dissected from adult flies at 3 days post-eclosion (A,C) and at 15 days post-eclosion (D,F). Scale bars: 30µm. Graphs represent quantification of the number of pericardial nephrocytes at 3 days (B) and 15 days (E) using >15 samples/genotype from 3 independent experiments. Statistical analysis was performed with Kruskal Wallis with Dunn's post-test. For all panels: ns, non significant, ***p<0.001 (See S1 Table for details on transgenic flies).



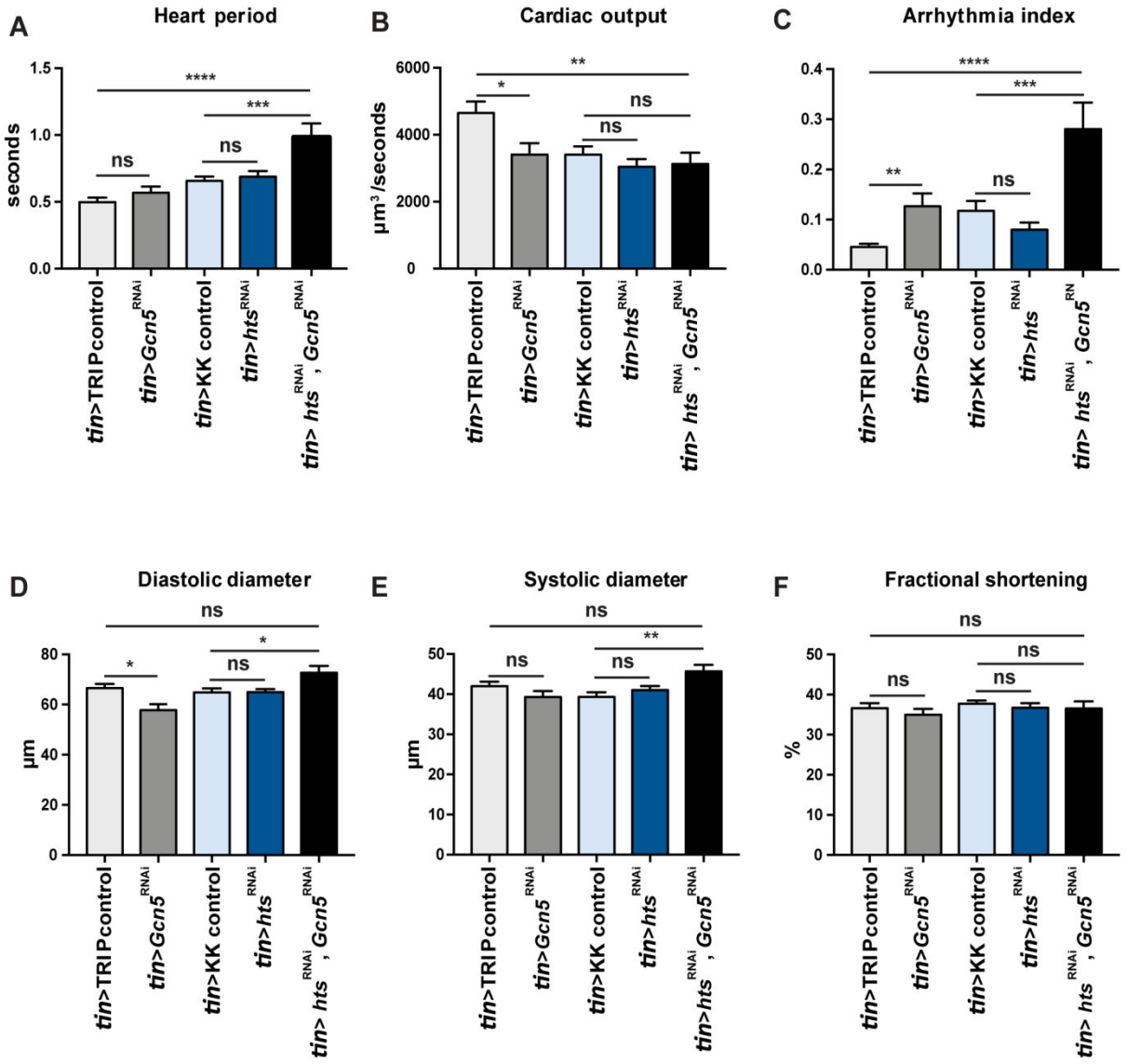
S9 Fig. Number of pericardial nephrocytes in adducin-αγ rescue animals

(A) Pericardial nephrocytes in adducin-αγ WT and E559Q rescue and control adult flies at 15 days post-eclosion were stained for the differentiation markers Kirre (red) and Pyd (blue). Scale bar: 30µm. **(B)** Quantification of the number of pericardial nephrocytes from n>8 samples/genotype in 3 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni's post-test. ns, non significant (See S1 Table for details on transgenic flies).



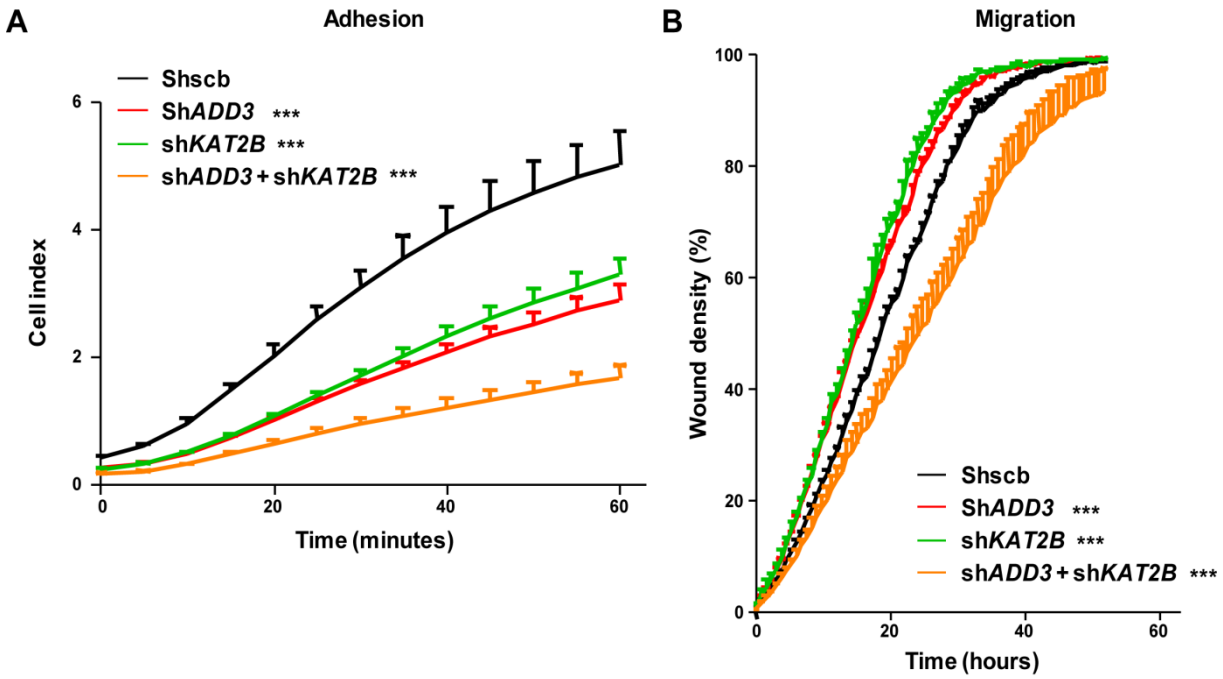
S10 Fig. Effect of adducin-αγ E559Q on *Drosophila* heart function

(A-C) M-mode of beating 2-week-old control (*yw/Df(2R);* A), adducin-αγ WT (B) and adducin-αγ E559Q (C) rescue hearts. Scale bar: 1 second. **(D-H)** High-speed movies of beating adducin-αγ WT, adducin-αγ E559Q rescue and control hearts were analysed using semi-automated Optical Heartbeat Analysis [45]. For quantification, 8-19 flies were analyzed. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison, except for Arrhythmia index (H; n=8-19, Mann-Whitney-Wilcoxon). For all panels: ns, non significant, ***p<0.001 (See S1 Table for details on transgenic flies).



S11 Fig. Effect of cardiac-specific depletion of *hts* and *Gcn5* on *Drosophila* heart function

(A-F) *Tin*-GAL4 driver was used to knockdown *hts* or/and *Gcn5* in cardiomyocytes, and different heart parameters were analyzed in 3 week-old adult flies. Two separate control RNAi lines (TRIP and KK) were used to match $Gcn5^{RNAi}$ (TRIP) and hts^{RNAi} (KK), respectively. For quantification, 19-30 flies were analyzed. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison for all parameters except arrhythmia index, which was analysed using Mann-Whitney-Wilcoxon. For all panels: ns, non significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



S12 Fig. Effect of ADD3 and/or KAT2B knockdown on human cultured podocytes adhesion and migration

(A) Adhesion was assessed using the xCELLigence system (ACEA Biosciences). Cells were plated with complete medium in the E-plate 96. Data obtained were analyzed with the RTCA software. Results are presented as time *vs.* cell index curve (n=3 independent experiments; linear regression analysis). **(B)** Migration was assessed using the Incucyte Scratch wound cell migration assay (Essen Bioscience). Cells were plated with complete medium 48 hours before scratch in ImageLock Plates-96 wells (Essen Bioscience) and images were recorded every 45 minutes after scratch until complete wound closure. Images were analyzed using the Incucyte Zoom software. Results are presented as percentage of wound cell density over time (n=3 independent experiments; linear regression analysis). For all panels: ns, non significant, *p<0.05 **p<0.01, ***p<0.001.

S1 Table. Fly strains used in this study

Mutants	**Origin**	***GAL4 drivers***	**Origin**
hts^{null}	Takashi Suzuki, University of Tokyo	*tub*-GAL*4*	BL#5138 BDSC
Df(2R)BSC26 (here referred to as *Df(2R))*	Jan Pielage, University of Kaiserslautern	*da*-GAL4	Clement Carré, Pierre et Marie Curie University
Gcn5^{E333st}	Clement Carré, Pierre et Marie Curie University	*pros*-GAL4	Barry Denholm, Center for Integrative Physiology, Edinburgh
Df (3L)Sex 204 (*Df(3L))*	Clement Carré, Pierre et Marie Curie University	*Dot*-GAL4	Zhe Han, Children's Research Institute, Washington
Df(2R)247	BL#7155 BDSC	*Mhc*-ANF-RFP, *Hand*-GFP; *Dot*-GAL4	Zhe Han, Children's Research Institute, Washington
CG31030^{MI00107}	BL#30620 BDSC	*Tin*-GAL4	Manfred Frasch, Mount Sinai School of Medicine, New York
UAS-RNAis	**Origin**		
UAS-Hts RNAis (*Hts*^{RNAi}*)*	KK#103631 VDRC		
UAS-Gcn5 RNAis (*Gcn5*^{RNAi}*)*	BL#33981 BDSC		
UAS-TRPP2 RNAi (*TRPP2*^{RNAi}*)*	#6941 VDRC		

Abbreviations: BDSC, Bloomington *Drosophila* Stock Center; VDRC, Vienna *Drosophila* Resource Center. *hts*^{null} hemizygous flies were generated by crossing the *hts*^{null} flies with *Df(2R)BSC26* flies that carry a deletion overlapping the *hts* locus. Similarly, Gcn5^{null} hemizygous flies were generated by crossing *Gcn5*^{E333st} with *Df(3L)Sex204* flies that carry a deletion of *Gcn5* and two contiguous genes. For rescue experiments, the binary GAL4:UAS system was used to drive the expression of the rescue constructs ubiquitously or in the tissue of interest, on the *hts*^{null} *or Gcn5*^{null} hemizygous or KD backgrounds.

Summary of the findings and discussion

In this project we identified potential pathogenic homozygous missense variants in two candidate genes, *ADD3* (c.1975G>C, p.E659Q) and *KAT2B* (c.920T>C, p.F307S), in the affected individuals from a consanguineous family. These individuals presented borderline microcephaly and developmental delay, cataracts, skeletal defects, SRNS and dilated cardiomyopathy. The segregation analysis in non-affected family members did not exclude any mutation. *ADD3* encodes adducin γ, an actin capping protein that also links the submembranar spectrin skeleton to F-actin (Matsuoka, Li et al. 2000). *KAT2B* encodes the lysine acetyl transferase 2B (KAT2B), involved in acetylation of lysine residues on histone H3 and regulation of transcriptional programs (Nagy and Tora 2007). We initially proposed three hypotheses regarding the causality of the mutations 1) only one of the mutations is pathogenic and responsible for the disease phenotype 2) both mutations are pathogenic and contribute to the disease phenotype in a synergic way 3) both mutations are pathogenic but affect different organs, giving rise to the complex phenotype observed in the affected individuals.

About one year after we started our project, Kruer *et al.* described a consanguineous family, where another homozygous mutation in *ADD3* was found in affected individuals presenting a partially overlapping syndrome with the one found in our patients, with no SRNS or heart disease (Kruer, Jepperson et al. 2013). Using GeneMatcher (https://genematcher.org/) (Sobreira, Schiettecatte et al. 2015) we later found two additional families carrying biallelic mutations for *ADD3*, again exhibiting only neurological findings with facultative cataracts and very similar skeletal defects, but no evidence of SRNS or heart disease. These genetic data strongly support the causality of *ADD3* mutations for the neurological phenotype, skeletal defects and cataracts, but not for the cardiomyopathy or kidney disease, that we then hypothesized to be due to the *KAT2B* mutation.

In order to assess the pathogenicity of the mutations and define their individual contribution to the patient phenotype we performed functional assays *in vitro* and in the *Drosophila* model. In flies, we used already existent *null* alleles for the fly homologs of *ADD3* and *KAT2B*, and we then performed the rescue of the phenotype using the GAL4:UAS system. This system allowed us to ubiquitously express the respective human or fly genes in the null background. For each gene, comparison of the phenotype obtained using the cDNA WT or carrying the corresponding human mutations allowed us to define the pathogenicity of the mutations at the functional level. We have also performed the specific KD of the genes of interest in nephrocytes or myocardial cells, using cell specific drivers, namely *Pros*-GAL4 and *Dot*-GAL4 for nephrocytes and *Tin*-GAL4 for myocytes.

Patient fibroblasts from individuals carrying *ADD3* and *KAT2B* mutations showed reduced KAT2B protein levels despite equal mRNA levels, while ADD3 levels showed no significant difference. In *Drosophila*, rescue experiments using the WT or E659Q adducin γ showed that this mutation reduces fly viability and impairs adult fly motor function. However, no differences were seen in heart or nephrocyte function. On the other hand, rescue experiments for KAT2B showed that the corresponding human mutation strongly decreased fly viability and induced multiple morphological and functional defects, including heart and nephrocyte damage, as well as loss of H3K9 acetylation, a main KAT2B target. These results define the *ADD3* mutation as a hypomorphic variant with no impact on heart or kidney function and the *KAT2B* mutation as a stronger loss of function variant that could induce heart and kidney defects.

To address the possibility of additive or synergistic effect between *ADD3* and *KAT2B* mutations in the kidney or heart phenotypes, we simultaneously silenced both genes in *Drosophila* nephrocytes and heart. The simultaneous KD induced a more severe effect than the silencing of each gene alone. The same effect of ADD3 and KAT2B silencing was also observed for migration and adhesion in cultured podocytes.

Altogether, these results clearly prove that both *ADD3* and *KAT2B* mutations are deleterious and suggest that *KAT2B* pathogenic variants could be responsible for the heart and kidney phenotype in humans, either alone or on a susceptible genetic background, as conferred by the adducin mutation in this particular family.

In the next sections I will discuss the evidence for the causality of the mutations on the patient phenotype and the possible underlying mechanisms, as well as the pitfalls we encountered along the project.

ADD3 mutations

Including the patients identified by Kruer et al., a total of 4 unrelated families and 8 affected individuals harbor biallelic mutations in *ADD3*. All these individuals show central nervous system (CNS) disease, manifesting as mild to severe developmental delay and borderline or significantly reduced cephalic perimeter. In addition, some of the patients show epilepsy and corpus callosum defects at magnetic resonance imaging (MRI). Non CNS manifestations include cataracts and mild skeletal defects, present in two non-related families with *ADD3* mutations. All the patients were specifically assessed for the presence of proteinuria and kidney function, as well as heart disease by

echocardiogram. However except for the individuals that also have a *KAT2B* mutation, no proteinuria or cardiac disease was found. These human genetic findings strongly point to the causality of *ADD3* mutations in the CNS disease, cataracts and skeletal defects.

Adducin γ has a globular head region and a neck region, both responsible for the hetero-oligomerization with adducin α, and a tail region with a myristoylated alanine-rich C kinase substrate (MARCKS) related domain at its C-terminus that has homology with the MARCKS protein. The neck and tail region, particularly the MARCKS related domain, are necessary for the interaction with actin and spectrin and for the capping of F-actin barbed ends (Matsuoka, Li et al. 2000). All the *ADD3* mutations were phylogenetically conserved missense mutations that distributed along the head and tail regions of the protein. The missense homozygous mutation previously identified by Kruer et al. (G367D) induced a severe neurological phenotype, was localized at the neck region and was shown to reduce colocalization with adducin α, suggesting impaired interaction (Kruer, Jepperson et al. 2013). Interestingly patients carrying the homozygous N332S mutation, located in the head region, in the vicinity of the neck region, also had a severe neurological phenotype. The homozygous mutation E659Q in the tail region on the other hand, does not alter colocalization with adducin α (our data) and the patient's neurological phenotype is milder. This suggests that disruption of oligomerization could be responsible for the severity of the neurological manifestations. The patients that carry the biallelic N332S/ N29S,V530I mutations also have a milder neurological phenotype. The N29S and V530I mutations, altering aminoacids respectively localized in the head and the tail region of the protein, are in the same allele and both are rare missense variants, however, according to Polyphen and SIFT scores, V530I is probably more deleterious than N29S (N29S Polyphen 0.653 – possibly damaging, SIFT 0.25 – tolerated; V530I Polyphen 0.990 – probably damaging SIFT 0 – Damaging). However, as we have two different variants in different regions of the protein, it is hard to draw conclusions about their respective functional effect and contribution to the phenotype.

ADD3 is a paralog of *ADD1*, encoding adducin α, and *ADD2*, encoding adducin β, in humans. These genes originated by duplication events in vertebrates. Adducin proteins in mammals form heterotetramers composed of either adducin α and γ, expressed ubiquitously, or adducin α and β, expressed in specific brain regions and erythrocytes (Matsuoka, Li et al. 2000). In mouse models, *Add1* constitutive KO leads to lethal hydrocephalus in 50% of the mice, growth retardation, hemolytic anemia and strain dependent scoliosis and megaesophagus (Robledo, Ciciotte et al. 2008, Sahr, Lambert et al. 2009), while *Add2* KO shows erythrocyte defects (spherocytosis) and motor coordination and learning deficits (Gilligan, Lozovatsky et al. 1999). *Add3* KO on the other hand has no overt phenotype (Sahr, Lambert et al. 2009).

Adducin β expression has been shown to increase in the absence of adducinγ, and vice-versa, as a compensation mechanism that allows maintenance of functional adducin heterotetramers (Sahr, Lambert et al. 2009). These findings highlight a possible redundancy between adducin β and γ. However, these compensation mechanisms may be tissue and cell dependent, and we do not know to what extent the adducin γ functions are completely rescued by adducin β and vice-versa.

In *Drosophila* the homolog of all human adducins is *hts* that has multiple isoforms generated by alternative splicing (Whittaker, Ding et al. 1999, Petrella, Smith-Leiker et al. 2007). *Hts* deficiency in flies induces severe synaptic and viability defects, with most flies dying during 2nd instar stage (Ohler, Hakeda-Suzuki et al. 2011). Hypomorphic alleles show female sterility due to disturbed fusome formation and absence of F-actin rich ring canals in oogenesis (Yue and Spradling 1992, Petrella, Smith-Leiker et al. 2007). We observed that *hts null* flies also exhibited rough eyes and had severe motor defects. The poor conservation of the region where the E659 amino acid is located did not allow us to use the *Drosophila* hts in rescue experiments, we thus used the human adducins. Interestingly, we showed that rescue of the *hts null* fly viability defects was only achieved by expressing both human adducin α and γ (hereby abbreviated adducin-αγ). By immuno-fluorescence these proteins colocalized and expression of both isoforms increased the adducin γ levels, confirming what had been previously reported for mammals (Matsuoka, Li et al. 2000, Sahr, Lambert et al. 2009).

Rescue flies carrying the adducin-αγ with the E659Q mutation on the *hts null* background had a significant albeit small reduction in viability and showed motor defects that could be due to neuromotor impairment. However they were otherwise morphologically indistinguishable from the adducin-αγ WT rescue flies, had no rough eyes, and no defect was found on brain size. In nephrocytes, the absence of *hts* leads to nephrocyte defects already visible at the larval stage, namely on the subcellular localization of Kirre, but most evident at the adult stage, where specific KD in pericardial nephrocytes caused loss of differentiated nephrocytes in adult flies. However, rescue experiments using adducin-αγ, failed to show any difference between the WT forms and the E659Q mutation in larval garland nephrocytes or in pericardial nephrocytes in adult flies. These results show that although ADD3 E659Q mutation is pathogenic it clearly does not induce a complete loss of function, corresponding rather to a hypomorph allele.

Why would some tissues such as the eye and the nephrocytes show a phenotype with *hts* loss of function but then a complete rescue with this mutation is less clear. Several explanations could be proposed for this: 1) the mutation is hypomorphic and not severe enough to induce visible defects in nephrocytes or in the eye for instance 2) the nephrocytes could be less sensitive than neurons to

adducin γ loss of function due to the existence of other partially overlapping pathways that could compensate for this loss. Moreover, it should be noted that the *hts* deficiency in flies corresponds to the loss of all the three adducin paralogs in humans, and that this could indeed induce a podocyte defect, but that this would not be the case if you only loose adducin-γ.

To answer to these questions it would be interesting to see if missense mutations found in patients with more severe neurological manifestations, such as the p.N332S mutation, could induce a phenotype in nephrocytes (transgenic flies have been ordered to help address this point). Nevertheless, in all the patients mutated for *ADD3* alone, glomerular disease is not observed even in affected individuals with the most severe neurological phenotypes.

KAT2B mutations

The identification of a homozygous missense *KAT2B* mutation in addition to the ADD3 homozygous mutation in a family that has additional heart disease and SRNS suggested that this mutation could be responsible for these additional defects.

KAT2B, alternatively called P300/CBP associated factor (PCAF), forms homodimers in order to exert its functions (Shi, Lin et al. 2014). It has a PCAF homology domain that interacts with several binding partners, such as CBP/p300, an acetyl transferase domain, responsible for its enzymatic functions, and a bromodomain, that mediates interaction with histone tails (Nagy and Tora 2007). The mutation is localized in the PCAF homology domain, and could thus disrupt the binding to other partners. Similar to *ADD3*, *KAT2B* also has a paralog in vertebrates, *KAT2A*, encoding the lysine acetyl transferase 2A (KAT2A). The ubiquitous KO of *Kat2b* in mice induces short term memory defects (Maurice, Duclot et al. 2008) in young mice but no other overt phenotype. However, when these mice are challenged, for example in response to central nervous system injury or limb ischemia, an important role is seen for KAT2B in the response to injury and the regeneration process (Bastiaansen, Ewing et al. 2013, Puttagunta, Tedeschi et al. 2014). *Kat2a* KO mice, on the other hand, fail to form dorsal mesoderm and embryos die at 10.5 dpc (Xu, Edmondson et al. 2000), arguing for a crucial role of *Kat2a* in development. Double *Kat2a* and *Kat2b* KO mice die earlier, suggesting partially overlapping functions but not complete redundancy (Xu, Edmondson et al. 2000). KAT2B expression is enriched in the organs affected in patients, including kidney, heart and also muscle (Xu, Edmondson et al. 1998).

Interestingly mutations in the partner proteins of KAT2B, CBP and P300, lead to Rubinstein Taby syndrome, an autosomal dominant disease that is characterized by skeletal defects, neural development defects and cardiac and urogenital malformations (Petrij, Giles et al. 1995). No mutations in humans have been reported for its paralog gene *KAT2A*.

The homolog gene of *KAT2A* and *KAT2B* in flies is *Gcn5*. Loss of *Gcn5* in flies leads to a viability defect, most larvae dying during metamorphosis (Carre, Szymczak et al. 2005). The absence of rescue with the human WT forms of KAT2B and/or KAT2A, prevented us from using the human transgenes, so rescue experiments were performed using the fly gcn5, where the mutated amino acid was conserved (F307S corresponding in flies to F304S). Flies rescued with the WT Gcn5 had a complete rescue of the fly viability and showed no obvious defects, while those recued with the Gcn5 F304S mutation had altered overall fly morphology, were unable to fly and showed clear heart function and nephrocyte defects. Moreover acetylation of H3K9, one of the main Gcn5 substrates, was also clearly reduced, suggesting an impact on the overall protein function and classifying this mutation as a severe loss of function mutation.

These results clearly show a pathogenic role for the *Gcn5* F304S mutation in flies. However due to the existence of the *KAT2A* gene in mammals, and a possible degree of redundancy between both *KAT2A* and *KAT2B*, it is hard to know from the fly phenotype what would be the loss of function phenotype of *KAT2B* in humans. This would depend on the number of redundant functions with KAT2A, on the tissue expression level of both genes and on the tissue specific needs in response to noxious stimuli.

We have further shown that a human *KAT2B* homozygous missense variant found in a healthy individual from our in-house database (S502F) and predicted to be pathogenic by some but not all algorithms [Polyphen probably damaging (score=0.98), SIFT Damaging (score=0), SDM: increased protein stability) did not induce any phenotype in flies and completely rescued their viability defects (S502 corresponding in flies to S478). These results show that *in silico* predictions are not always correct and underline the importance of testing the pathogenicity of the mutations *in vivo*. If the mutation found in the healthy individual had a deleterious phenotype in *Drosophila Gcn5*, we would have concluded that KAT2B was redundant in humans. However as this was not the case, these results confirm the pathogenicity of the F307S mutation but do not allow us to conclude whether KAT2B is redundant or not in humans. Several *in vitro* studies in mammalian cells as well as in mouse models attribute singular and non-redundant functions to KAT2B. I.e. in mammalian cells specific KAT2B silencing has been shown to block miogenic differentiation (Puri, Sartorelli et al. 1997), modulate p53 activity (Linares, Kiernan et al. 2007, Love, Sekaric et al. 2012) and stress responsive

cell cycle-arrest (Love, Sekaric et al. 2012) and to have a role in telomere maintenance (Jeitany, Bakhos-Douaihy et al. 2017). Moreover, in *Kat2b* -/- mouse models most studies reveal that KAT2B plays a role in the regenerative process after injury (Bastiaansen, Ewing et al. 2013, Puttagunta, Tedeschi et al. 2014). This is concordant with what has been shown for other proteins that modulate epigenetic mechanisms in terminally differentiated cells such as the podocyte (see chapter "Epigenetic regulation in podocytes"). Moreover, in the patients carrying *KAT2B* mutations it is interesting to note that there is a relatively late onset of the kidney and heart diseases. In this regard, it would be interesting to see if *Kat2b* KO mice challenged with a specific noxious stimulus or during ageing develop SRNS or heart disease.

Synergic/additive effect between *ADD3* and *KAT2B* loss of function mutations?

To investigate if there is a synergic/additive effect between *ADD3* and *KAT2B* mutations that would be responsible for a phenotype in heart and kidney, we silenced both *ADD3* and *KAT2B* homolog genes in flies and in a human podocyte cell line. Although the silencing of each gene alone showed defects in nephrocyte maintenance as well as in migration and adhesion in cultured podocytes, we found that the silencing of both genes was significantly more deleterious than each gene alone. For heart function, only the silencing of *Gcn5* but not *hts* leads to a phenotype. However, the simultaneous KD of both genes had a more deleterious effect than *Gcn5* alone. This argues in favor of an additive effect between both mutations in nephrocytes/podocytes and a synergistic effect in heart. Unfortunately, we could not directly assess the possibility of synergy between both *ADD3* and *KAT2B* mutations, as this would need a double *hts* and *Gcn5 null* background to re-express the Gcn5 and the adducin α,γ WT or mutated forms. This would be technically very difficult to obtain using our GAL4:UAS strategy.

We can nevertheless conclude that the *ADD3* mutant background may favor the appearance of the *KAT2B* mutation phenotype in humans. However, these data do not allow us to exclude that the *KAT2B* mutation alone could be enough to produce the disease phenotype in humans, as it is the case in the *Drosophila* model.

Interestingly Shaw *et al*. found that *KAT2B* rare variants of undefined pathogenicity are enriched in patients with sporadic FSGS. In a mouse model with genetic susceptibility for FSGS (see "Genetic susceptibility factors and oligogenism" chapter), the silencing of KAT2B in podocytes led to a trend towards increased proteinuria, although this did not reach significant values (Yu, Artomov et al.

2016). Moreover, when kidney sections from these mice were analyzed by electron microscopy, dispersed areas of podocyte foot process effacement were found (Yu, Artomov et al. 2016). This could mean that indeed deleterious mutations in *KAT2B* may manifest in a genetic susceptibility background such as the ADD3 mutated background. It would thus be interesting to see the effect of the double KO of *Add3* and *Kat2b* in mice.

KAT2B mutations on the other hand do not seem to aggravate the neurologic manifestations, cataracts or skeletal defects of *ADD3* mutations, as these were of identical severity in patients carrying heterozygous compound biallelic *ADD3* mutations and no *KAT2B* mutations *vs* patients with both *ADD3* and *KAT2B* mutations.

Possible pathogenic mechanism mediated by ADD3 loss of function

Most of the research on adducin function has been performed in neurons. In axons adducins have been shown to stabilize actin ring structures that distribute periodically and maintain the axon diameter (Xu, Zhong et al. 2013, Leite, Sampaio et al. 2016). It also stabilizes synapses and the neuromuscular junctions (Pielage, Bulat et al. 2011). Interestingly, in neurons adducin is able to interact with transmembrane proteins and link them to the underlying actin cytoskeleton (Ohler, Hakeda-Suzuki et al. 2011, Wang, Yang et al. 2011).

Due to the crucial functions of the cytoskeleton in podocyte biology, theoretically these cells could be affected by mutations on adducin γ or this could create a susceptible background for further injury by KAT2B loss of function. In fact, it is feasible that adducin may also serve as an adaptor protein between the components of the slit diaphragm and the underlying actin cytoskeleton. Indeed, adducin has also been shown to interact with erythrocyte sub-membranar proteins (Franco, Chu et al. 2016), particularly stomatin (Innes, Sinard et al. 1999), a protein from the same family as podocin. It is also known to functionally interact with anillin (Wang, Chadha et al. 2015), recently found to be mutated in SRNS (Gbadegesin, Hall et al. 2014). It would thus be interesting to test interactions of podocyte specific proteins with adducin γ. This could be done by performing co-immunoprecipitation of adducin γ followed by mass spectrometry, ideally in isolated mouse glomeruli or podocytes.

Possible pathogenic mechanisms mediated by KAT2B loss of function in podocytes

KAT2B is able to modulate transcriptional programs through the acetylation of histones and transcription factors (Nagy and Tora 2007). Moreover it has been shown to have intrinsic ubiquitination activity (Linares, Kiernan et al. 2007), modulate PTEN activity through acetylation (Okumura, Mendoza et al. 2006) and being implicated in cellular responses to stress through the modulation of p53 and p21 (Liu, Scolnick et al. 1999, Chao, Wu et al. 2006, Love, Sekaric et al. 2012). Moreover, KAT2B has been shown to drive myogenic differentiation through the interaction and acetylation of MyoD (a bHLH transcription factor), one of the main transcription factors that drives muscle differentiation (Sartorelli, Puri et al. 1999, Dilworth, Seaver et al. 2004, Kuninger, Wright et al. 2006). Interesting TCF21, also called Pod1, another bHLH transcription factor, has been shown to be crucial for podocyte differentiation in mice, and *Tcf21* KO in podocytes leads to proteinuria and FSGS (Maezawa, Onay et al. 2014). We could thus imagine that KAT2B drives alterations in the transcriptional profile of podocytes that could lead to altered expression of podocyte specific proteins and dedifferentiation. In this regard, it would be interesting to see if KAT2B interacts with or acetylates TCF21 or the podocyte main transcription factor WT1. We actually addressed this question by performing co-immunoprecipitation experiments between tagged KAT2B and TCF21 over-expressed proteins. Our preliminary data indicated a possible interaction, although not modified by the presence of the mutation.

It is interesting to note that Gcn5 silencing led to the loss of differentiated pericardial nephrocytes, as seen by the reduction of nephrocytes showing Kirre or Pyd expression in 15 day-old flies. Moreover, these cells also lose the GFP expression driven by Hand, another bHLH transcription factor, important for pericardial nephrocytes and heart development (Han and Olson 2005, Han, Yi et al. 2006). However, this could be a general nephrocyte phenotype in response to injury as occurs in the *hts* KD, as well as in the KD of the CD2AP homolog *Cindr*, or in the *Klf15* transcription factor *null* allele (our finding and others (Ivy, Drechsler et al. 2015, Na, Sweetwyne et al. 2015)).

Besides the known epigenetic mechanisms, KAT2B loss of function could also lead to altered acetylation of several other proteins besides histones or transcription factors. In fact, recently, a proteomic approach in *KAT2A/KAT2B* KD cells revealed that a high number of proteins participating in cytoskeleton regulation and known to be crucial for podocyte function are indeed acetylated by the KAT2A/B proteins (Fournier, Orpinell et al. 2016). Examples include α-actinin-4 and TTC21B known to be mutated in FSGS (Fournier, Orpinell et al. 2016). In this regard, ADD3 and KAT2B could finally belong to a common pathway, converging on the cytoskeleton of podocytes.

Conclusion of *ADD3* and *KAT2B* project

Using the *Drosophila* model we clearly showed the pathogenicity of the mutations found in *ADD3* and *KAT2B*. Even if in humans the disease manifested quite late in childhood, the fact that in flies we had very clear and easily quantifiable read-outs, such as the fly viability, readily observable exterior phenotypic defects (rough eyes, defects in wings, locomotor defects), as well as the number of nephrocytes in adult flies, made it relatively easy to assess the impact of the mutations. However, one limitation of this project was the fact that both adducin and KAT2B have several paralogs and just one homolog in flies, which did not allow us to clearly define what would be the affected organs in humans. Nevertheless, the combination of the human genetics and functional data suggest that *ADD3* mutations are responsible for the neurological and skeletal manifestations, as well as cataracts, and that *KAT2B* mutations induce defects in heart and podocytes, either alone or in a susceptible genetic background that in the case of our family would be due to *ADD3* mutations.

Conclusion

Conclusion

An important lesson of this work is that the *Drosophila* is an excellent model for addressing the pathogenicity of mutations in new candidate genes. In fact, if the loss of function of the fly homolog gene induces a phenotype, such as decreased viability, external morphological defects or locomotor defects, this makes it very easy and fast to assess the impact of the mutations by comparing the WT rescue phenotype with that of the mutant. In fact, in both projects of this thesis we have been able to clearly define the pathogenicity of the mutations found in *SGPL1*, *ADD3* and *KAT2B* using this strategy. Interestingly, the fly model was even able to discriminate between more severe and less severe *SGPL1* mutations, and this corresponded to the phenotype observed in patients. Underlining the accuracy of the fruit fly for defining the pathogenicity of mutations found in humans, a mutation that was shown to be benign by other functional tests (E132G found in *SGPL1*) behaved similarly to the WT in the multiple read-outs we have used in the fruit-fly. Moreover, the *KAT2B* homozygous variant found in a healthy individual also gave no phenotype in flies. We can thus imagine a potential application of this tool for the cases where the pathogenicity of the mutations poses a challenge, in new or even well-known SRNS genes.

Assessment of the impact of the mutations in nephrocyte structure and function, although more cumbersome than a viability assay due to the time-consuming process of organ dissection, imaging and quantification, is also feasible. Several read-outs that we and others have used, such as albumin filtration/endocytosis and immunofluorescence for proteins of the slit-diaphragm, have been studied by two different groups and have provided quite reliable results. Assessment of nephrocyte foot process effacement by TEM, on the other hand, can give a qualitative idea of the phenotype, but the time and cost of quantification of foot process effacement is probably too high to be done in the routine testing of candidate gene mutations. As we found that the silencing of *ADD3* and *KAT2B* homologs in flies led to a decrease in the number of differentiated nephrocytes in ageing adult flies, it would be interesting to see if this is a common nephrocyte response to injury, in which case it could also constitute an easily quantifiable read-out, especially if studying late onset SRNS.

Another main conclusion of our work is based on the issue of the degree of genomic redundancy in humans *vs* flies. In fact, while the lower genomic redundancy of flies has the advantage of simplifying loss of function studies and of providing easy read-outs, such as the viability and widespread defects in multiple organs, the disadvantage is that when a single gene in flies has multiple homolog genes in humans, it may be difficult to extrapolate from the fly phenotype the consequence of the loss of function of only one of the paralog genes in humans, as they may have acquired particular tissue

specificity or divided their ancestral functions between them. This was in fact the main issue with the ADD3/KAT2B project. In this case complementation of the *Drosophila* experiments with other vertebrate models would be of great value.

In this thesis we have focused on assessing the causality of mutations found in new SRNS candidate genes, however the *Drosophila* model is without any doubt an excellent tool for further exploring the underlying mechanisms of the disease as well as possible modifier pathways. Moreover, large scale drug assays are also feasible in fruit flies, although possible limitations related to the route of administration, bioavailability, as well as unknown kinetics in flies must be considered. .

While podocyte-related research has long been focused in the cytoskeleton and slit diaphragm of these cells, genetic studies in recent years have brought to light the importance of other organelles and cellular pathways. In this thesis we highlight two novel mechanisms that may open new avenues for better understanding podocytopathies. Firstly the specific importance of the sphingolipids for the podocyte, where we can hypothesize an effect on lipid rafts, the effect of bioactive sphingolipid signaling or even cellular crosstalk mechanisms. Secondly our work on KAT2B highlights the impact of epigenetics on podocyte biology, as well as the general role of protein acetylation in the podocytopathies.

Finally, the second project of this thesis exemplifies the complexity of medical genetics in the era of NGS, with the existence of very rare mutations in one or few families greatly hindering the establishment of a correct genetic diagnosis. Even if competition between research groups is a reality, this issue certainly highlights the importance of sharing rare and private variants with other scientific groups and in online databases such as Genematcher. In our case, this was crucial to define the contribution of *ADD3* mutations to the patient phenotype.

I hope that in the future the *Drosophila* model will progressively take a relevant role in medical genetics and in the kidney field as this little animal has much to offer in defining the pathogenicity of mutations, the mechanism of the disease and even potential therapeutic strategies.

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