Department of Pathology
Faculty of Medicine
University of Helsinki
Finland

THE ROLES OF CD2AP AND SHIP2 IN INSULIN RESISTANCE AND PODOCYTE APOPTOSIS

Tuomas Aleksi Tolvanen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the Lecture Hall 2 at Haartmaninkatu 3, on 18th December 2017, at 12 noon.

Helsinki 2017

Supervised by

Assistant Professor Sanna Lehtonen
Department of Pathology
University of Helsinki
Helsinki, Finland

Reviewed by

Professor Seppo Vainio
Faculty of Biochemistry and Molecular Medicine
University of Oulu
Oulu, Finland

and

Professor Jukka Hakkola
Research unit of Biomedicine
University of Oulu
Oulu, Finland

Official opponent

Professor Richard Coward
Bristol Medical School (THS)
University of Bristol
Bristol, United Kingdom

ISBN 978-951-51-3904-7 (paperback)
ISBN 978-951-51-3905-4 (PDF)
http://ethesis.helsinki.fi
Unigrafia Oy
Helsinki 2017

To my wish for a better world

ABSTRACT

Background: Type 2 diabetes, characterised by insulin resistance, is an ever-increasing problem in the world. Diabetic nephropathy is a renal microvascular complication of diabetes that is a common cause of end-stage renal disease worldwide. Low levels of albumin can be detected in the urine at an early stage of diabetic nephropathy, and more severe albuminuria develops as the disease progresses. Insulin resistance is associated with albuminuria in patients with type 1 or type 2 diabetes. Also, the loss of podocytes plays a major role in the pathogenesis of diabetic nephropathy. The mechanisms underlying the development of insulin resistance and apoptosis in podocytes are still not fully understood. This study aimed to investigate the pathophysiological mechanisms leading to insulin resistance and podocyte apoptosis at the molecular level, and concentrated to the roles of CD2-associated protein (CD2AP) and SH2-domain containing 5' inositol phosphatase 2 (SHIP2) in these processes.

Results: We found that the lack of CD2AP in podocytes led to attenuated glucose uptake, without affecting PI3K-AKT-mediated insulin signalling. This led us to investigate the role of CD2AP in glucose transporter trafficking. Indeed, live-cell imaging revealed that internalized Glucose transporter 4 (GLUT4) was trapped in the perinuclear region of podocytes lacking CD2AP. CD2AP co-fractionated with known components of GLUT4 vesicles and interacted with clathrin and clathrin adaptor GGA2. These results suggest that CD2AP plays a role in GLUT4 vesicle trafficking.

We further observed that the level and activity of SHIP2, an interaction partner of CD2AP, was increased in the absence of CD2AP in podocytes. Also, production of reactive oxygen species (ROS) and the rate of apoptosis were increased when CD2AP was lacking from podocytes. We hypothesized that inhibition of SHIP2 would decrease the production of ROS and apoptosis, but even though we detected reduced ROS production, inhibition of SHIP2 increased podocyte apoptosis in the absence of CD2AP.

In the search for novel SHIP2 inhibitors we found an old anti-diabetic drug, metformin, to bind to and inhibit the activity of SHIP2 *in silico*, *in vitro* and *in vivo*. By inhibiting the activity of SHIP2, metformin increased glucose uptake and protected podocytes from apoptosis initiated by SHIP2 overexpression. SHIP2 exhibited higher activity in the kidney pieces received from nephrectomy patients with type 2 diabetes receiving non-metformin medication compared to nephrectomy patients without diabetes; in

comparison, the activity of SHIP2 did not differ between metformin receiving patients with type 2 diabetes and people without diabetes. Patients with type 2 diabetes with metformin medication also showed reduced podocyte loss.

Conclusions: In the light of our results we suggest a novel role for CD2AP in the regulation of sorting of internalized GLUT4 and regeneration of insulin responsive GLUT4 vesicle compartment. We also suggest that SHIP2 inhibitors, including metformin, can be used to reduce the oxidative stress of podocytes and to prevent podocyte loss, except in the case of patients suffering from reduced expression of CD2AP.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I Tuomas A. Tolvanen, Surjya Narayan Dash, Zydrune Polianskyte-Prause, Vincent Dumont, Sanna Lehtonen. Lack of CD2AP disrupts Glut4 trafficking and attenuates glucose uptake in podocytes. Journal of Cell Science 128, 4588-4600, 2015.

II Pauliina Saurus*, Tuomas A. Tolvanen*, Sonja Lindfors, Sara Kuusela, Harry Holthöfer, Eero Lehtonen, Sanna Lehtonen. Inhibition of SHIP2 in CD2AP-deficient podocytes ameliorates reactive oxygen species generation but aggravates apoptosis. Scientific Reports. Accepted.

III Zydrune Polianskyte-Prause*, Tuomas A. Tolvanen*, Sonja Lindfors, Mervi Latvala, Hong Wang, Surjya N. Dash, Mika Berg, Vincent Dumont, Harry Nisen, Tuomas Mirtti, Per-Henrik Groop, Kristiina Wähälä, Jukka Tienari, Sanna Lehtonen. Metformin enhances insulin sensitivity and protects against kidney injury by inhibiting SHIP2 activity. Submitted.

* equal contribution

ABBREVIATIONS

AER	albumin excretion rate
AMPK	AMP-activated protein kinase
CAP	c-Cbl associated protein
CCP	clathrin coated pit
CD2AP	CD2-associated protein
CDK2	cycling-dependent kinase 2
COPI	coat protein I
DN	diabetic nephropathy
ER	endoplasmic reticulum
ERC	endosomal recycling compartment
ERK	extracellular-signal related kinase
FFA	free fatty acid
GBM	glomerular basement membrane
GFB	glomerular filtration barrier
GFP	green fluorescent protein
GGA	Golgi-localized gamma-ear containing ADP-ribosylation factor binding protein
GSV	GLUT4 storage vesicle
GLUT4	glucose transporter 4
HA	hemagglutinin
HTS	high-throughput screening
IC_{50}	inhibitory concentration 50
IGF	insulin-like growth factor
IL-6	interleukin 6
IR	insulin receptor
IRAP	insulin-responsive amino peptidase
IRS-1/2	insulin receptor substrate ½
KO	knockout
MAPK	mitogen-activated protein kinase
PA	puromycin aminonucleoside

PCK1	phosphoenolpyruvate carboxykinase 1
PDK1	3-phosphoinositide dependent kinase 1
$PI(3,4)P_2$	phosphatidylinositol(3,4)bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol(3,4,5)trisphosphate
PI3K	phosphoinositide 3-kinase
PM	plasma membrane
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
SD	slit diaphragm
SHIP2	SH2-domain-containing inositol 5'-phosphatase 2
TDZ	thiazolidinedione
TGF-β	transforming growth factor beta
TGN	trans-Golgi network
TNF	tumor necrosis factor
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
WT	wild type

1 INTRODUCTION

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes or adult onset diabetes, is an ever-growing problem in the world. In 2015, there were 415 million patients with diabetes of whom 90% had T2DM, and the number of people with diabetes is estimated to increase to 640 million by the year 2040 (International Diabetes Federation, 2015). Annual diabetes-related expenditure is 673 billion US dollars, but more importantly, 5 million people suffer from premature death every year due to diabetes and diabetes-related complications. Poor management of the blood glucose level increases the risk of developing micro- and/or macrovascular complications. Microvascular complications include diabetic nephropathy (DN), retinopathy and neuropathy, whereas cardiovascular disease, stroke and peripheral vascular disease are classed as macrovascular complications (Zimmet *et al.*, 2001).

Insulin resistance of the tissues is a characteristic feature of T2DM. Insulin resistant state manifests as reduced activity of the canonical PI3K insulin signalling pathway visualized as reduced AKT phosphorylation and attenuated glucose uptake in response to insulin (Kahn *et al.*, 1992; Maianu *et al.*, 2001; Xiong *et al.*, 2010). In addition to its function in regulating glucose homeostasis, insulin functions as a growth factor, thus affecting cell survival (Avruch, 1998; Pronk *et al.*, 1993). Insulin resistance has been associated with kidney injury in both T1DM and T2DM patients (Martin *et al.*, 1992), and insulin sensitizers have been suggested to have a renoprotective role in patients with diabetes (Miyazaki *et al.*, 2007). In the kidney glomerulus, podocytes are the only cells responsive to insulin (Coward *et al.*, 2005) and undergo apoptosis if insulin signalling is abolished (Welsh et al, 2010).

The level of CD2-associated protein (CD2AP) in podocytes has been shown to be downregulated in diabetic conditions (Ha et al, 2015). This ubiquitously expressed adaptor and scaffolding protein interacts with molecules involved in insulin signalling pathway and vesicle transport (Huber *et al.*, 2003; Schiffer *et al.*, 2004; Kobayashi *et al.*, 2004; Havrylov *et al.*, 2008; Wasik *et al.*,2012). Mice lacking CD2AP develop severe kidney injury and die of renal failure (Shih *et al.*, 1999), and cultured podocytes are more susceptible to apoptosis in the absence of CD2AP (Schiffer *et al.*, 2004).

Src homology 2 domain containing inositol 5'-phosphatase 2 (SHIP2), an interaction partner of CD2AP (Hyvönen *et al.*, 2010), is a negative regulator of the insulin signalling pathway (Ishihara *et al.*, 1999). The level of SHIP2 has

been shown to be upregulated in rodent models of T2DM (Hyvönen *et al.*, 2010; Hori *et al.*, 2002). The overexpression of SHIP2 downregulates the activation of AKT and induces apoptosis in several cell types (Gorgani-Firuzjaee *et al.*, 2015; Hyvönen *et al.*, 2010; Soeda *et al.*, 2010). The insulin sensitivity of L6 myotubes and diabetic mice can be improved by inhibiting the activity of SHIP2 by using small-molecule inhibitors (Suwa *et al.*, 2009; 2010b).

Insulin resistance and increased apoptosis of podocytes play a critical role in the pathogenesis of DN. Hence, deeper knowledge of the molecular mechanisms underlying insulin resistance and apoptosis in podocytes is essential to better understand the pathophysiological mechanisms of DN and to develop novel treatments for it. This study was initiated by the findings that CD2AP interacts with SHIP2 (Hyvönen *et al.*, 2010) and that glucose uptake is reduced and the level of SHIP2 upregulated in podocytes lacking CD2AP.

We found that lack of CD2AP in podocytes abrogates insulin-induced glucose uptake due to a dysfunction in sorting of GLUT4-containing vesicles and reformation of insulin responsive GLUT4 vesicle compartment. Lack of CD2AP in podocytes also increased the level and activity of SHIP2, reactive oxygen species (ROS) generation and apoptosis. The phenotype was rescued by re-introducing CD2AP back to podocytes, but inhibiting SHIP2 activity only reduced the level of ROS without rescuing podocytes from apoptosis in the absence of CD2AP. In search for novel SHIP2 inhibitors we found that an old anti-diabetic drug metformin inhibits SHIP2 activity and prevents podocyte apoptosis *in vitro* and *in vivo*. These results suggest that CD2AP and SHIP2 play an important role in insulin resistance and apoptosis in podocytes, and propose that novel SHIP2 inhibitors could be used in the treatment of insulin resistance and DN.

2 REVIEW OF THE LITERATURE

2.1 Kidney structure and function

Kidneys (Figure 1. A) are symmetrically located on both sides of the abdominal cavity of a vertebra. The main function of kidneys is to act as waste management facilities by filtering plasma and secreting waste products from blood to urine. They play also a vital role in many biological functions from regulation of the blood pressure to hormonal secretion, and sustaining fluid, electrolyte, and acid-base homeostasis in the body.
The functional unit of a kidney is called nephron (Figure 1. B) and each healthy human kidney has approximately one million of them. Nephrons are composed of two main structures: glomeruli and tubuli. A glomerulus consists of a capillary tuft, which is located inside of the Bowman's capsule. Glomeruli are localized in the cortex of the kidneys. Plasma is continuously filtrated in the glomeruli forming approximately 180 litres of primary urine per day. The tubular system forms the distal part of the nephron, and it passes from the kidney cortex deep into the medulla of the kidney. The tubular system is divided into the proximal tubule, Loop of Henle and distal tubule, that is connected to the collecting duct. In the tubular system, most of the water and electrolytes are re-absorbed so that normal urine excretion is approximately 1 to 1.5 litres per day.

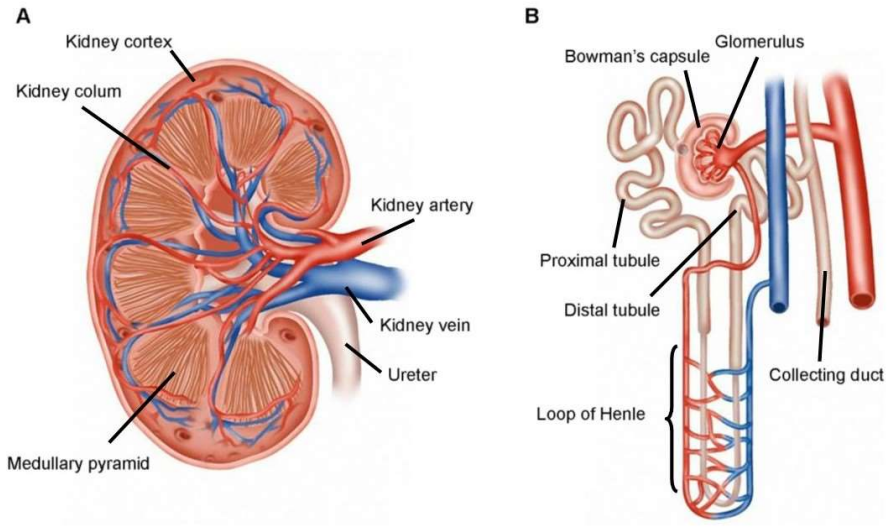

Figure 1. Structure of (A) kidney and (B) a nephron. Modified from Wellcome Images.

2.2 Glomerular filtration barrier

Glomerulus (Figure 2.) consists of a capillary vessel tuft that is surrounded by the Bowman's capsule. Blood flowing to the capillary vessels is filtered through the glomerular filtration barrier (GFB) into the urinary space (Figure 2B). Only water and molecules smaller than albumin pass through the GFB and larger molecules remain in the blood. The GFB has three layers: fenestrated epithelial cells, glomerular basement membrane (GBM) and podocytes. In addition to being a physical size selective barrier, the GFB provides also charge selectivity due to the negative charges of glycocalyx of the epithelium and anionic proteins of the GBM.

The first part of the GFB is the endothelium that lines the inner surface of the glomerular capillaries. The endothelial cells are fenestrated, meaning that they exhibit numerous trans-cellular holes of 70-100 nm (in humans). Glycocalyx, consisting of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids, covers the luminal side and fenestrations of the endothelial cells. As it is negatively charged, it acts as the first line for the charge selectivity of the GFB (Jeansson and Haraldsson, 2006). The endothelium synthesizes laminin and type IV collagen thus being an essential element in the formation of the GBM (Abrahamson *et al.*, 2009; John and Abrahamson, 2001).

The GBM is an extracellular structure, 300-350 nm thick, located in between the endothelial cells and podocytes. Both cell types contribute to the formation and maintenance of the GBM. In addition to laminin and type IV collagen, the GBM consists of nidogen and heparan sulphate proteoglycans. These proteoglycans exhibit an anionic charge thus forming the second line of the charge selective barrier (reviewed by Haraldsson *et al.*, 2008).

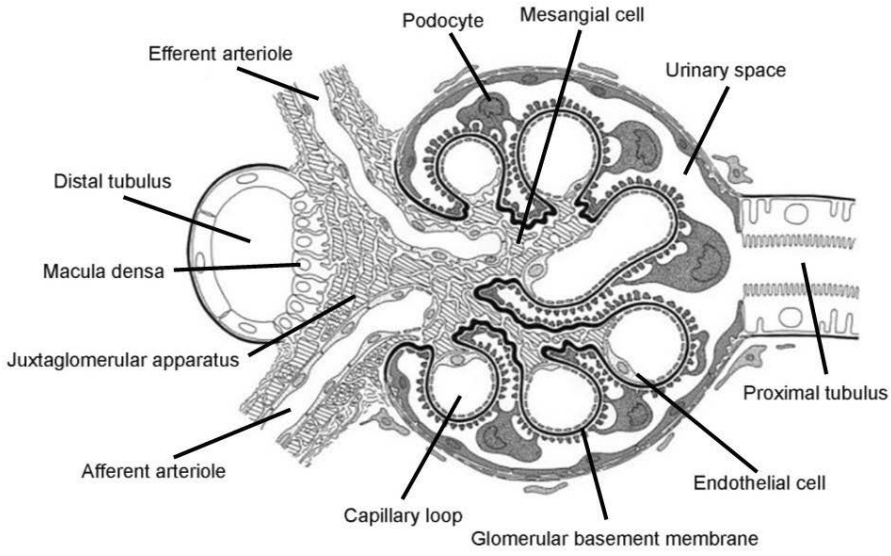

Figure 2. Structure of the glomerulus. Modified from (Kriz *et al.*, 1998).

Glomerular visceral epithelial cells, also known as podocytes, are terminally differentiated and highly specialized cells covering the whole surface of the GBM. Their cell body gives rise to major protrusions that split to smaller protrusions called foot processes. Podocytes form unique 40 nm-wide zipper-like cell-cell adhesions between foot processes, called slit diaphragms (SD). The pores formed by SDs are roughly the size of albumin and thus the SDs are thought to be the sieve preventing albumin and larger proteins from leaking into the urine (Rodewald and Karnowsky 1974), while remaining highly permeable to water and other small molecules.

2.3 Diabetes

Diabetes is a disease characterized by hyperglycaemia, which results from a defect in insulin secretion, insulin action or both. Insulin is a small peptide hormone and one of the most potent anabolic hormones. It is produced and secreted by pancreatic β-cells in the islets of Langerhans. Insulin-stimulated glucose uptake in muscle and adipose tissues and inhibition of hepatic glucose production are essential to the regulation of blood glucose concentration and to energy storage. Insulin has also a pivotal role in cell growth, differentiation and survival (reviewed by Saltiel and Kahn, 2001; Pessin and Saltiel 2000).

Type 1 diabetes mellitus (T1DM) is an autoimmune disease in which pancreatic β-cells producing insulin are destroyed, thus leading to the need of insulin replacement therapy. T1DM is usually an early onset disease diagnosed mainly in children.

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes or adult onset diabetes, is an ever-growing problem in the world. Insulin resistance of the tissues, a characteristic feature of T2DM, is often a result of obesity. As the body weight gets higher, the more insulin needs to be secreted by the pancreas leading to chronically elevated level of circulating insulin. As a result, the insulin sensitivity of the tissues diminishes and pancreas needs to produce more insulin to keep the blood glucose at the normal level, leading ultimately to the fatique of the β-cells and diminished insulin secretion. The mechanisms leading to insulin resistance can vary from genetic to environmental factors but the phenotype is still similar: reduced activity of the canonical PI3K insulin signalling pathway manifested as reduced AKT phosphorylation and attenuated glucose uptake in response to insulin.

Poor management of the blood glucose level leads to glucotoxicity that increases the risk of developing micro- and/or macrovascular complications, which gradually progress over extended period of time. Microvascular complications include diabetic nephropathy (DN), retinopathy and neuropathy, whereas cardiovascular disease, stroke and peripheral vascular disease are classified as macrovascular complications.

2.3.1 Insulin resistance and factors contributing to its development

Insulin resistance is a state where the normal circulating concentration of insulin hormone is not enough to transduce signal to a physiological response. This can be due to defects in the insulin signalling pathway and its regulation, glucose transporter trafficking, or both (Kahn *et al.*, 1992; Maianu *et al.*, 2001; Xiong *et al.*, 2010).

Several altered metabolic states can lead to insulin resistance, among them elevated blood glucose, circulating free fatty acids (FFA), and cytokine and insulin levels (Pessin and Saltiel, 2000). At the cellular level, persistent hyperglycaemia leads to excess intracellular glucose that induces production of advanced glycation endproducts, which impairs canonical insulin signalling pathway (Miele *et al.*, 2003). Glucotoxicity together with hyperinsulinemia is associated with hyperphosphorylation of IRS proteins, which may lead to chronic desensitization to insulin (Paz *et al.*, 1997; Potashnik *et al.*, 2003). Oxidative stress derived from mitochondrial dysfunction and ER stress is also

increased in hyperglycaemic conditions (Fiorentino *et al.*, 2013). In addition to hyperglycaemia, also an elevated level of FFAs stimulates ROS production. Furthermore, elevated FFAs attenuate insulin signalling and insulin-stimulated glucose uptake (Roden *et al.*, 1996). T2DM patients, but also obese non-diabetic subjects (Stepien *et al.*, 2014), usually exhibit chronic low-level inflammation and proinflammatory cytokines, such as TNF-α and IL-6 secreted by adipocytes, are known to promote peripheral insulin resistance (Bastard, *et al.*, 2006; Plomgaard *et al.*, 2005). Excess of TNF-α alone causes insulin resistance in the skeletal muscle of healthy subjects demonstrated by attenuated phosphorylation of AKT and impaired glucose uptake (Plomgaard *et al.*, 2005). TNF-α and IL-6 also promote a vicious circle by increasing the release of FFAs (Plomgaard *et al.*, 2008), very low-density lipoprotein and inducing hypertriglyceridaemia (Nonogaki *et al.*, 1995).

2.4 Diabetic nephropathy

DN is the leading cause of kidney disease in patients with T1DM or T2DM eventually requiring renal replacement therapy, such as kidney transplantation or dialysis. It affects around 40% of the patients suffering from T1DM or T2DM (Gross *et al.*, 2005; Zimmet *et al.*, 2001).

Table 1. *Animal models utilized to study diabetic kidney disease*

Genetic modification	Strain	Descripiton	Reference
Akita	C57BLKS and FVB	T1DM, very mild kidney phenotype develops at 30 weeks	Wang *et al.*, 1999
db/db	C57BLKS	T2DM, kidney phenotype develops at 8-16 weeks	Like *et al.*, 1972
E1-DN	FVB	T1DM, kidney phenotype develops at 10-20 weeks	Hyvönen *et al.*, 2015
fa/fa	Zuker rat	T2DM, kidney phenotype develops at 18 weeks	Zucker *et al.*, 1972
MKR	FVB	T2DM non-obese, kidney phenotype develops at 10-30 weeks	Mallipattu *et al.*, 2014
ob/ob	BTBR	T2DM, very mild kidney phenotype develops over time	Hudkins *et al.*, 2010
OLETF	rat	T2DM, kidney phenotype develops at 22-30 weeks	Kawano *et al.*, 1994

Ove26	FVB	T1DM, kidney phenotype detected at 16-24 weeks	Epstein *et al.*, 1989
podIRKO	C57BL/6, 129/SV and FVB	Podocyte-specific knock-down of insulin reseptor, kidney phenotype at 8 weeks	Welsh *et al.*, 2010

To model type 1 diabetes mice can be treated with streptozotosin, which destroys the beta-cells of pancreas. The susceptibility of streptozotosin-treated mice to develop kidney phenotype depends of the strain of the mice (Betz and Conway, 2014). These chemically induced or genetic models of diabetes do not fully mimic human DN, but are currently the best tools available.

2.4.1 Definition and clinical features of diabetic nephropathy

As DN is a progressive kidney disease, it is categorized into different stages based on the albumin excretion rate (AER). Microalbuminuria is the first stage characterized by AER of 30-300 mg/24 h. In the second stage, macroalbuminuria, AER is >300 mg/24 h. Proteinuria, that is the final stage, is characterized by an AER >500 mg/24 h. It takes years to develop macroalbuminuria and proteinuria, leading to a decrease in estimated glomerular filtration rate (eGFR< <60 ml/min/1.73 m2). Both are considered as a definition of more advanced disease (Reutens and Atkins, 2011).

Typically, microalbuminuria is detected 5-15 years after the diabetes is diagnosed. Increase in AER over time predicts the progression from microalbuminuria to proteinuria, within 6-14 years, in 80% of patients with T1DM (Viberti *et al.*, 1982; Mogensen and Christensen, 1984). As the treatment of hyperglycaemia and hypertension of patients with T1DM and T2DM has progressed, the risk of progression has decreased so that 30-40% of patients progress only to macroalbuminuria, instead of proteinuria, within the next 10 years (Adler *et al.*, 2003; Caramori *et al.*, 2000; Forsblom *et al.*, 1992). However, some of the patients with microalbuminuria may regress back to normoalbuminuria (Perkins *et al.*, 2003).

2.4.2 Glomerular pathology in diabetic nephropathy

DN is characterized by the presence of specific histological and structural changes in the kidney. The development of the changes starts early in the course of diabetes and changes progresses as the disease develops. The thickening of the GBM and mesangial expansion are the starting points in the glomerular classification of DN, which may lead to advanced diabetic glomerulosclerosis (Tervaert *et al.*, 2010). Even though the thickening of the GMB is the characteristic early finding in T1DM and T2DM patients with DN,

it does not correlate with the severity of the disease (Mauer *et al.*, 1984; White and Bilous, 2000). Mesangial expansion is the hallmark of diabetic nephropathy in both T1DM and T2DM patients (Mauer *et al.*, 1984; White and Bilous, 2000). It is divided into mild and severe stages depending on the expanded area caused by increased extracellular material in the mesangium (Tarveart *et al.*, 2010). The changes in podocytes include foot process widening (Bjorn *et al.*, 1995) and effacement, loss of slit diaphragms (reviewed in Mundel and Shankland, 2002), and podocyte loss due to apoptosis (Susztak *et al.*, 2006). The effacement of podocyte foot processes and the decreased number of podocytes strongly predicts the progression of DN (Toyoda *et al.*, 2007; Weil *et al.*, 2012). As the number of podocytes per glomeruli decreases, the total surface area covered by podocytes does not change (Pagtalunan et al, 1997). Being terminally differentiated cells, podocytes depend on hypertrophy to increase their mass and size in glomeruli to cover the same area as before (Wiggins *et al.*, 2005). The changes occuring in DN on the level of tissue and cells in relation to the clinical stage of the patients is reviewed in detail by Tervaert *et al.* and Najafian *et al.* (Tervaert *et al.*, 2010; Najafian *et al.*, 2015).

2.4.3 Tubular aspects of diabetic nephropathy

In addition to glomerulus, also tubulur compartment actively participates to the progression and development of diabetic kidney disease (Vallon *et al.*, 2011). Excess amount of circulating glucose leaks through the glomerulus to the tubulus and causes extra stress to the renal proximal tubule epithelial cells (RPTEC) responsible for reabsorbing glucose from the primary urine (Vallon *et al.*, 2011). As these cells are not able to absorb all the glucose it ends up to the distal nephron segments. These segments do not come in contact with glucose in normal conditions and start accumulating glycogen in the cells (Dombrowski *et al.*, 2007). This change can be detected already after 1 week after the induction of diabetes in mice (Gatica *et al.*, 2015). The diabetes-induced renal damage has been suggested to occur first in the distal nephron and then to extend to the proximal tubules (Kang *et al.*, 2005). Albumin, which is a well-known pro-inflammatory and pro-fibrotic factor, starts to leak to tubulus as the glomerulus becomes damaged and RPTEC try to compensate this by increasing its absorbtion (Vallon *et al.*, 2011; Gosmanov *et al.*, 2014). When the leakage of albumin becomes overwhelming, it ends up to the distal nephron segment and to the urine (Vallon *et al*, 2011).

2.4.4 Podocyte apoptosis and insulin resistance

Apoptosis of podocytes increases significantly after the onset of hyperglycaemia and correlates with the onset of albuminuria in T1DM Akita mice and T2DM db/db mice. Increased extracellular concentration of glucose stimulates the generation of reactive oxygen species (ROS) in cultured

podocytes *via* mitochondrial pathways and NADPH, and activates proapoptotic p38 MAPK and caspase-3 (Susztak *et al.*, 2006). Also transforming growth factor beta (TGF-β) can induce MAPK-mediated apoptosis and loss of podocytes (Schiffer *et al.*, 2001). Lack of CD2AP has also been shown to make podocytes more susceptible to apoptosis (Schiffer *et al.*, 2004).

Insulin resistance is also associated with DN in patients with T1DM or T2DM (Orchard *et al.*, 2002; Parvanova *et al.*, 2006), and it correlates with the severity of albuminuria (Orchard *et al.*, 2002; Ekstrand *et al.*, 1998). Podocytes are insulin sensitive cells (Coward *et al.*, 2005), and their insulin resistance was first reported in db/db mice, animal model mimicking T2DM (Tejada *et al.*, 2008). In a similar fashion, PI3K-mediated insulin signalling was shown to diminish in the glomeruli of T2DM Zucker rats (Mima *et al.*, 2011). Insulin appears to be a critical signalling molecule for the podocytes, as the podocyte-specific knockout of insulin receptor from mice (podIRKO) demonstrates: Effacement of foot processes was detected at the age of 5 weeks, and the mice started developing albuminuria. At the age of 8 weeks, podIRKO mice exhibit classical signs of DN, such as thickening of the GBM, and loss of foot processes and podocytes due to significantly increased podocyte apoptosis (Welsh *et al.*, 2010). The knockout of IR from podocytes abrogated the PI3K and MAPK signalling pathways, proving that these pathways are truly activated by insulin (Welsh *et al.*, 2010). In addition to insulin, insulin-like growth factor II (IGF-II) activates PI3K and MAPK pathways in podocytes and protects them from apoptosis (Hale *et al.*, 2013). Overexpression of SHIP2 has also been reported to increase podocyte apoptosis (Hyvönen *et al.*, 2010). The connection between microalbuminuria and insulin resistance has been reported in diabetic (Parvanova *et al.*, 2006; Yip *et al.*, 1993) and non-diabetic (Palaniappan *et al.*, 2003) patients, suggesting that insulin resistance provokes podocyte apoptosis.

From the aspect of the glucose transporter trafficking, it appears that nephrin is essential for the insulin responsive glucose uptake to podocytes (Coward *et al.*, 2007). Several studies have reported that the expression of nephrin is downregulated in patients with DN (Kim *et al.*, 2007; Langham *et al.*, 2002; Baelde *et al.*, 2004) and in experimental models of DN (Hyvönen *et al.*, 2015; Wu *et al.*, 2008).

2.5 Key proteins linked to the insulin sensitivity of podocytes

Podocytes express various membrane and cytosolic proteins which are paramount for the function and maintanence of the filtration machinery of the

kidney. The SD protein nephrin and cytosolic proteins CD2AP, SHIP2 and septin 7 are linked to insulin signalling, actin remodelling and glucose transporter trafficking in podocytes (Coward *et al.*, 2007; Hyvönen *et al.*, 2010; Wasik *et al.*, 2012).

2.5.1 Nephrin

Nephrin is a paramount protein for the SD (Holthöfer *et al.*, 1999; Ruotsalainen *et al.*, 1999), and regulates actin dynamics (Jones *et al.*, 2006) as well as insulin-induced glucose transporter trafficking (Coward *et al.*, 2007). It protects podocytes against apoptosis by activating the PI3K pathway (Huber *et al.*, 2003), and in addition, the reduction of mitochondrial oxidative stress protects podocytes from apoptosis (Daehn *et al.*, 2014). Nephrin binds to adaptor protein CD2AP, which connects nephrin to the actin cytoskeleton (Lehtonen *et al.*, 2002).

2.5.2 CD2AP

CD2-associated protein (CD2AP) is a ubiquitously expressed cytosolic adapter protein harboring three SH3-domains, a proline rich domain and a coiled-coil domain (Dustin *et al.* 1998; Kirsch *et al.*, 1999; Li *et al.*, 2000). It was first found in T-cells of the immune system (Dustin *et al.*, 1998), and thus it was unexpected that CD2AP knockout mice died at the age of 6 to 7 weeks from renal failure, phenotypically resembling human congenital nephrotic syndrome of the Finnish type (Shih *et al.*, 1999) that is caused by mutations in the gene encoding nephrin (Kestilä *et al.*, 1998). In the kidney, CD2AP is mainly expressed in podocytes and is a vital component of the slit diaphragm as it connects nephrin, the primary component of the SD, to the actin cytoskeleton (Shih *et al.*, 1999; Li *et al.*, 2000; Lehtonen *et al.* 2000; Lehtonen *et al.*, 2002).

Further studies have shown that CD2AP functions as a scaffolding protein in signalling pathways and in vesicle trafficking (Huber *et al.*, 2003; Schiffer *et al.*, 2004; Kobayashi *et al.*, 2004; Havrylov *et al.*, 2008; Wasik *et al.*, 2012). In cellular signalling, CD2AP is known to interact with the regulatory subunit p85 of phosphosinositide-3' kinase (PI3K) and is involved in PI3K-dependent AKT activation, protecting cells from transforming growth factor beta (TGF-β)-induced apoptosis (Huber *et al.*, 2003; Schiffer et al, 2004). In 2005, Welsh *et al.* showed that CD2AP regulates the assembly of actin on vesicles and is thus involved in endosomal sorting/trafficking. It has also been found to interact with the active form of the small GTPase Rab4 (Cormont *et al.*, 2003) and to co-localize with coat protein I (COPI) vesicles and clathrin (Havrylov *et al.*, 2008). Further, the CD2AP-/- mice were found to have a defect in the

formation of the multivesicular bodies implying that CD2AP has a role also in the degradation pathway (Kim *et al.*, 2003). Our group has shown that CD2AP directly interacts with Src homology 2 domain containing inositol 5'-phosphatase 2 (SHIP2) (Hyvönen *et al.*, 2010), a key regulator of the insulin signalling pathway (Ishihara *et al.*, 1999).

2.5.3 SHIP2

Ubiquitously expressed SHIP2 contains an N-terminal SH2-domain and 5'-phosphatase domain, a proline rich region, and a sterile alpha motive (SAM) domain in its C-terminus (Pesesse *et al.*, 1997; Zhuang *et al.*, 2007). As SHIP2 has many motifs for protein-protein interactions, it is recruited to receptor complexes through the binding to adaptor and scaffolding proteins or by binding directly to receptors *via* its SH2-domain. This allows the enzyme to hydrolyse its substrates at specific locations on the membranes. SHIP2 acts as a negative regulator of PI3K-mediated insulin signalling (Hyvönen *et al.*, 2010; Suwa *et al.*, 2010b) by hydrolysing phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P3) to phosphatidylinositol(3,4)bisphosphate (PI(3,4)P2) in various cell types (Pesesse *et al.*, 1998). SHIP2 is also found in clathrin-coated pits (CCP) where it regulates the maturation of CCPs by changing the phosphoinositolphospate concentrations on the membrane (Nakatsu *et al.*, 2010).

Knocking out SHIP2 gene, *Inppl1*, from mice does not alter the glucose or insulin tolerance of the mice, but makes them highly resistant to diet-induced weight gain. Still, the *Inppl1*-/- mice show lower insulin, cholesterol and triglycerides levels compared to wild type mice (Sleeman *et al.*, 2005). Mice expressing a catalytically inactive form of SHIP2 show similar phenotypical effects as described above (Dubois *et al.*, 2012). SHIP2 overexpression in mice leads to impairment of glucose tolerance and insulin sensitivity compared to WT littermates (Kagawa *et al.*, 2008). Insulin-induced AKT phosphorylation is also decreased in the tissues of the mice overexpressing SHIP2. Interestingly, these mice did not show any changes in cholesterol or triglyceride levels compared to WT mice, only increased insulin levels, indicating insulin resistance (Kagawa *et al.*, 2008). In humans, polymorphisms in *INPPL1* gene, encoding SHIP2, have been reported to associate with the metabolic syndrome and T2DM (Ishida *et al.*, 2006; Kagawa *et al.*, 2005; Kaisaki *et al.*, 2004) and the metabolic syndrome in male patients with T1DM (Hyvönen *et al.*, 2012).

2.6 Insulin signalling

2.6.1 PI3K-mediated pathway

In the canonical insulin signalling pathway (Figure 3.), binding of insulin to the insulin receptor (IR) activates autophosphorylation of IR on its tyrosine residues. This leads to increased catalytic activity of the tyrosine kinase, which phosphorylates the tyrosine residues of insulin receptor substrate family (IRS1/2) proteins. Phosphorylated IRS proteins interact with the SH2-domains of the p85 regulatory subunit of phosphatidylinositol 3' kinase (PI3K). PI3K is then activated and translocated to the plasma membrane (PM). Active PI3K utilizes $PI(4,5)P_2$ to produce $PI(3,4,5)P_3$, that acts as secondary messenger and activates phosphoinositide-dependent kinase 1 (PDK1) which phosphorylates T308 (Alessi *et al.*, 1997) of AKT and mTORC2 responsible for the phosphorylation of S473 of AKT (Sarbassov *et al.*, 2005). Fully active AKT that is phosphorylated on residues T308 and S473 then inactivates the Rab GAP AKT substrate of 160 kDa (AS160 also known as TBC1D4) and TBC1D1 *via* their phosphorylation (Klip *et al.*, 2014). This leads to increased GTP loading of their target Rab GTPases initiating the translocation of GLUT4 to the PM (Klip *et al.*, 2014).
SHIP2 and phosphatase and tensin homolog (PTEN), containing 5' and 3' phosphatase activities, respectively, act as the negative regulators of the PI3K pathway. Both of these enzymes target $PI(3,4,5)P_3$ and hydrolyse either the 3' phosphate group, producing $PI(4,5)P_2$, or the 5' phosphate group, producing $PI(3,4)P_2$. TAPP1 and 2 bind to the $PI(3,4)P_2$ produced by SHIP2 and recruit proteins to negatively regulate the phosphorylation of AKT (Wullschleger *et al.*, 2011).

2.6.2 CAP/c-CBL pathway

An alternative insulin signalling pathway is known as CAP (c-Cbl-associated protein)/c-Cbl pathway. In this pathway, c-Cbl is recruited to IR by CAP. Phosphorylation of c-Cbl initiates the translocation of CAP/c-Cbl complex to the lipid rafts and recruits CrkII-C3G complex also to lipid rafts. C3G then activates small GTP-binding protein TC10, which activates the translocation of GLUT4 to the PM (Reviewed in Staltiel and Kahn, 2001).

2.6.3 MAPK pathway

In insulin activated mitogen-activated protein kinase (MAPK) pathway (Figure 3.), SHC binds to activated IR and undergoes tyrosine phosphorylation. This leads to recruitment of GRB2, which then associates with SOS activating the Ras-ERK1/2 pathway (Pronk *et al.*, 1993).

Extracellular-signal related kinase (ERK) 1/2 is also known as p44/p42 MAPK. This signalling pathway does not regulate GLUT4 translocation, but contributes predominantly to cell growth and differentiation (Avruch, 1998). Interestingly, continuous activation of ERK though insulin-like growth factor I (IGF-I) receptor can lead to cell death (Sperandio *et al.*, 2004).

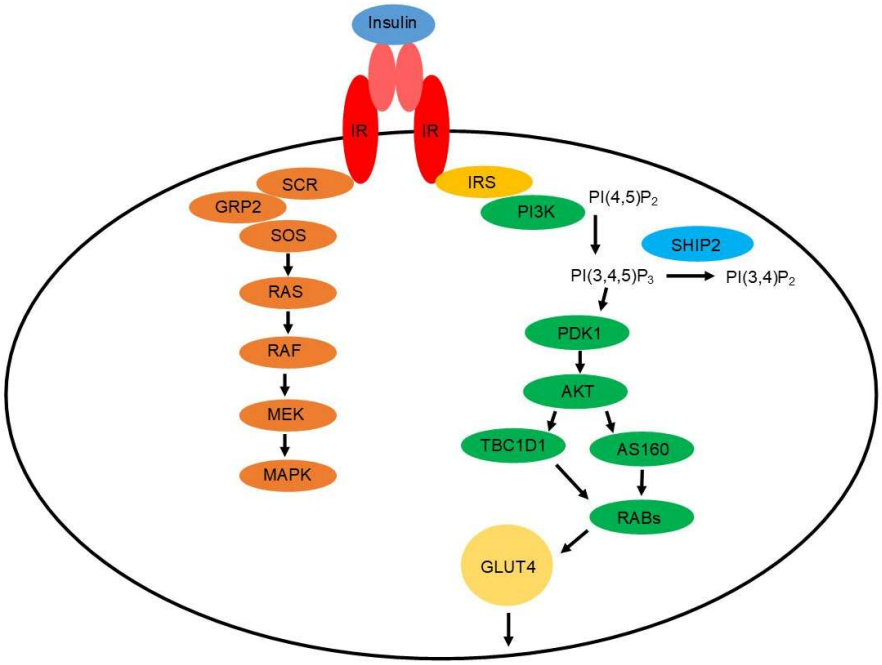

Figure 3. Insulin signalling pathways in podocytes.

2.6.4 Insulin signalling in podocytes

Like in muscle cells and adipocytes, insulin activates the PI3K pathway in podocytes, and thus, podocytes are also insulin sensitive cells (Coward *et al.*, 2005). In podocytes insulin also activates the MAPK signalling, which is involved in proliferation, transcriptional regulation and production of cytokines instead of metabolic actions (reviewed by Tian *et al.*, 2000). CAP/cCbl pathway does not function in podocytes or muscle cells, but is active in adipocytes (Welsh *et al.*, 2010). Insulin triggers the trafficking of glucose transporters to the plasma membrane in podocytes (Coward *et al.*, 2005) similarly as in other insulin sensitive cells.

2.7 Glucose transporters in podocytes

There are 14 known members in the glucose transporter family, but GLUT1, GLUT2, GLUT3, GLUT4, and GLUT8 are expressed in glomerulus and only GLUT1, GLUT4 and GLUT8 in podocytes (Coward *et al.*, 2005; Schiffer *et al.*, 2005; Lewko and Stepinski, 2009; Yan *et al.*, 2012). GLUT1 and GLUT4 localize to cytoplasm and plasma membrane of the podocytes, but the sub-cellular localization of GLUT8 is not known (Coward *et al.*, 2005; Yan *et al.*, 2012).

2.7.1 GLUT1

GLUT1 is mainly responsible for insulin independent facilitative glucose uptake, but it has been reported to translocate to the plasma membrane also in response to insulin in adipocytes (El-Jack *et al.*, 1999) and podocytes (Coward *et al.*, 2005). Podocyte-specific overexpression of GLUT1 in diabetic db/db mice protects the mice against mesangial expansion and accumulation of fibronectin (Zhang *et al.*, 2010).

2.7.2 GLUT4

GLUT4 is the predominant insulin-induced glucose transporter. After synthesis in the endoplasmic reticulum (ER), it is transported to the trans-Golgi-network (TGN). From the TGN it is sorted dynamically to generate GLUT4 storage vesicles (GSVs) (Figure 4.). In the absence of insulin most of the GLUT4 locates to the perinuclear region of the cells populating the GSV compartment, but a bit of GLUT4 can be found in the recycling endosomes. Vesicles in the GSV compartment are enriched with sortilin, and contain also insulin-responsive amino peptidase (IRAP), which is found in all the cytosolic compartments harbouring GLUT4 (reviewed in Kandror and Pilch, 2011). The question whether GSVs are under dynamic or static retention is largely under debate, as results from different studies support either of the two models (Bogan *et al.*, 2012; Fujita *et al.*, 2010; Martin *et al.*, 2006; Govers *et al.*, 2004). In mucle and adipose tissue, approximately 20% and 50% of the vesicles in the GSV compartment are transported to the PM in response to acute insulin stimulation (Figure 4.). Still, in addition to its translocation from the intracellular vesicle compartments to the PM, GLUT4 also needs to be activated on the membrane before it can take up glucose (Funaki *et al.*, 2004).

From the PM GLUT4 is internalized by either clathrin-dependent or -independent, or cholesterol-dependent endocytosis (Blot and McGraw, 2006) to early endosomes (Figure 4.). When insulin concentrations remain high, GLUT4 recycles directly back to the PM (Figure 4). In the absence of insulin,

early endosomes are incorporated into the endosomal recycling compartment (ERC), and GLUT4 is actively sorted back to the GSVs or to the TGN *via* retrograde transport (Blot and McGraw, 2006) (Figure 4.).

The whole-body knockout of GLUT4 in mice causes hyperinsulinemia and insulin resistance (Katz *et al.*, 1995). Interestingly, the glucose tolerance of these GLUT4-null mice is not altered when compared to control mice; this is apparently due to higher expression levels of other glucose transporters (Katz *et al.*, 1995). In normoalbuminuric human patients with diabetes, GLUT4 is upregulated in podocytes, but when microalbuminuria develops, the expression of GLUT4 plummets. The podocyte-specific knockout of GLUT4 in mice affects the nutrient sensing of podocytes but unexpectedly, protects them from the defects caused by diabetic conditions (Guzman *et al.*, 2014).

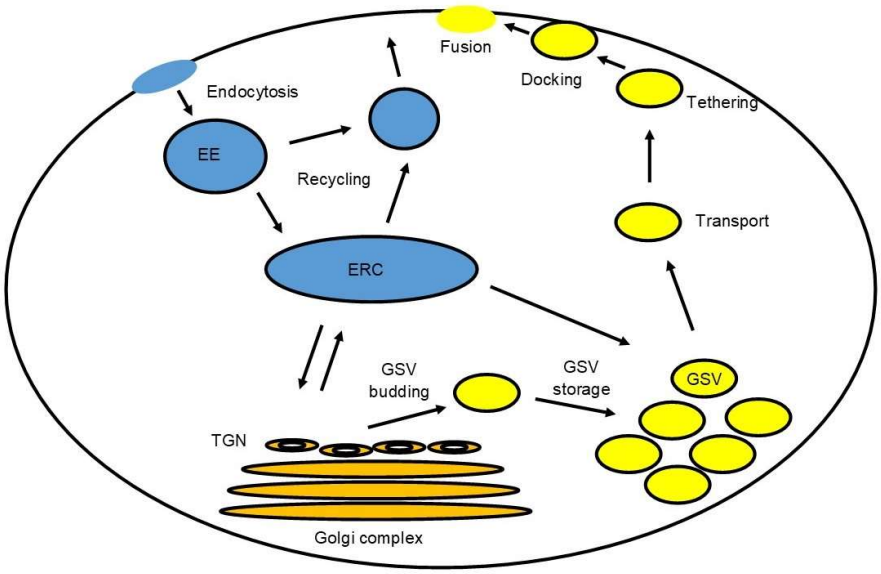

Figure 4. Trafficking of GLUT4. EE: early endosome; ERC: endosomal recycling compartment; GSV: GLUT4 storage vesicle; TGN: trans-Golgi network

2.8 Methods of drug discovery

Choosing the method for discovering novel drugs depends on the a priori knowledge of the target protein and molecules binding to it.

2.8.1 High-throughput screening

High-throughput screening (HTS) is a method that is often used if there are no known molecules binding to the target and the protein structure of the target is not known. In HTS, huge libraries of synthetized chemicals are biologically tested against the desired target. The goal is to identify compounds that show inhibition or activation capacity against the target. These molecules are called "leads" and are used as scaffolds to design novel molecules with better inhibition capacities and pharmacokinetic and other drug-like properties (Höltje *et al.*, 2003).

2.8.2 Ligand-based virtual screening

Ligand-based virtual screening methods can be used when there are some molecules that are known to bind to the target, but the 3D protein structure of the target is not yet resolved. The common structural and physicochemical properties of the known molecules are used as a template and molecular libraries are screened to find molecules with equivalent properties. The molecules used as these templates can be hits from the HTS. There are several methods for performing ligand-based virtual screening and they are extensively reviewed in (Höltje *et al.*, 2003, Tolvanen, 2008).

2.8.3 Structure-based virtual screening

Structure-based virtual screening methods can be used when the 3D structure of the target protein is available. The structure of the protein can be obtained through X-ray chrystallography or by using computational protein modelling. In this method, virtual libraries of small drug-like molecules are docked to the active site of the protein and scored by their computational interaction/binding energies. It is essential to identify the exact active site of the protein and to use that for the screening, as the protein structures are huge in relation to the size of the binding pocket, and it is not feasible to try to dock tens of millions of molecules over the entire protein.

2.9 Insulin sensitising drugs and small molecules

Insulin sensitizers are used in the treatment of T2DM as they deal with the core problem of the disease – insulin resistance. Insulin sensitizers in clinical use fall into two sub-classes: biguanides and thiazolidinediones (TDZs), also known as glitazones. Interestingly, the drugs do not directly affect canonical PI3K-AKT-mediated insulin signalling pathway, but they induce other signalling pathways to increase the glucose uptake to cells and suppress gluconeogenesis (Madiraju *et al.*, 2014; Turban *et al.*, 2012; Lehmann *et al.*,

1995). Inhibition SHIP2 has been hypothesized to be a good method to enhance the activity of the PI3K-AKT mediated pathway (Sasaoka *et al.*, 2006). Novel small molecules inhibiting SHIP2 have been identified using different drug discovery methods as summarized below.

2.9.1 Metformin

Metformin (Figure 5.) is the oldest and the only drug in the biguanide class that is still in use and recommended as a first-line treatment for patients with T2DM (Foretz *et al.*, 2014). Clinically used daily dosage of metformin for T2DM patients is 500-3000 mg (20 mg/kg/day). The mean plasma half-life of metformin is about 20 h, and as it is not metabolized in the body it is excreted to urine as such. The the peak plasma concentration of metformin depends on the dose but is between 10-18 μM (Foretz *et al.*, 2014). Metformin increases insulin sensitivity and glucose uptake in peripheral tissues (Foretz *et al.*, 2014), but reduction of gluconeogenesis in the liver is considered as its primary function (Hundal *et al.*, 2000). Metformin is a highly investigated drug and its mechanism of action has been suggested to be *via* activation of AMPK (Minassian *et al.*, 1998; Yuan *et al.*, 2002), inhibition of mitochondrial respiratory chain complex I (Viollet *et al.*, 2012), and most recently, by inhibition of mitochondrial glycerophosphate dehydrogenase (mGPD) in liver (Madiraju *et al.*, 2014). Interestingly, all the previously mentioned studies concerning the mechanism of action of metformin have concentrated on liver, but not on peripheral tissues, leaving the mechanism of action by which metformin increases insulin sensitivity elusive.

In the kidneys of patients with T2DM metformin has been reported to reduce albumin uria and high-glucose-induced podocyte apoptosis (Langer *et al.*,2016). Recently metformin was reported to protect podocytes of type 2 diabetic rats by increasing the expression of nephrin (Zhai *et al.*, 2017). It also protects podocytes against high-glucose-induced insulin resistance by preventing reduction SIRT1 protein level and by activating AMPK in rat podocytes (Rogacka *et al.*, 2017). Recently metformin has also been shown to modify gut microbiota composition and thus the abundance of the small molecules secreted by these bacteria in humans (Forslund *et al.*, 2015). The most common adverse effects of metformin are gut related, and this can also be due to the increase of *E. coli* (Forslund *et al.*, 2015).

2.9.2 Thiazolidinediones

The mechanism of action of TDZs is to activate nuclear peroxisome proliferator-activated receptors (PPARs), with the highest specificity towards PPARγ (Lehmann *et al.*, 1995). The main effect of TDZs is to reduce the

amount of circulating FFAs that forces cells to use glucose as their main source of energy (reviewed by Chen *et al.*, 2017). Pioglitazone (Figure 5.) is the only drug of this class that is still on the market in Finland. It has been withdrawn from the French and German markets as some studies have suggested that pioglitazone increases the risk of bladder cancer, but this is highly debated (Hampp and Pippins, 2017). Rosiglitazone was withdrawn from the European market as studies suggested that it increases the risk of cardiovascular events (Delea *et al.*, 2003).

2.9.3 SHIP2 inhibitors

The rationale behind SHIP2 inhibitors is to increase insulin sensitivity of the cells by blocking the catalytical activity of SHIP2. This ameliorates the signal transduction from PI3K *via* $PI(3,4,5)P_3$ to AKT as the inhibition of SHIP2 attenuates the production of $PI(3,4)P_2$ from $PI(3,4,5)P_3$. The published SHIP2 inhibitors fall into three categories: phosphorylated polyphenols (Vandeput *et al.*, 2007), thiophene-based SHIP2 inhibitors (Suwa *et al.*, 2009; and 2010b), and other heterocyclic structures (Annis *et al.*, 2009; Ichihara *et al.*, 2013).

Using 3D modelling, the researchers found that phosphorylated phenols resemble highly phosphoinositols. This led to the finding that phosphorylated polyphenols inhibit SHIP2, but are not very specific towards it. The best of these was biphenyl(2,3',4,5',6)P_5 (Figure 5.) with an IC_{50} value of 1,8 μM, but the activity data against SHIP1, a close homologue of SHIP2, or PTEN, a 3' phosphatase, was not reported (Vandeput *et al.*, 2007), leaving open the specificity of the inhibitor.

Two thiophene-based SHIP2 inhibitors, AS1949490 (Suwa *et al.*, 2009) (Figure 5.) and AS1938909 (Suwa *et al.*, 2010b) (Figure 5), were discovered by high-throughput screening at Astellas Pharmaceuticals. They have high potency against SHIP2 (IC_{50} approximately 0,60 μM) and do not inhibit SHIP1 (IC_{50} 13-21 μM) or PTEN (IC_{50} >50 μM). Both inhibitors increased AKT phosphorylation and glucose uptake and consumption in L6 myotubes. 10-day treatment of db/db mice with AS1949490 reduced the fasting plasma glucose levels and ameliorated oral glucose tolerance (Suwa *et al.*, 2009). The downfall of thiophene-based inhibitors is their poor solubility, poor pharmacokinetic properties, and limited cell permeability (Suwa *et al.*, 2010a). AS1949490 is commercially available and has been used in several studies mainly to alter the $PI(3,4)P_2$ concentrations effecting the interplay between actin and plasma membrane (Baranov *et al.*, 2016; Hughes *et al.*, 2015; Sharma *et al.*, 2013; Yoshinaga *et al.*, 2012).

Other heterocyclic molecules inhibiting SHIP2 were also found by using high throughput screening coupled with mass spectrometry in Neogenesis Pharmaceuticals (Annis *et al.*, 2009). In this study, the researchers studied only the effects of the inhibitor using purified SHIP2 and did not reveal any data on cultured cells or animals. Pyridine-based SHIP2 inhibitor (CPDA) (Figure 5.) was developed by ligand-based drug design using the published structures from Astellas and Neogenesis (Ichihara *et al.*, 2013). In this study, the activity of the molecule was studied only by its ability to induce AKT phosphorylation, and no data on the specificity or inhibition capacity against SHIP2 were shown. Still, CPDA did improve fasting blood glucose and the glucose and insulin tolerance of db/db mice (Ichihara *et al.*, 2013).

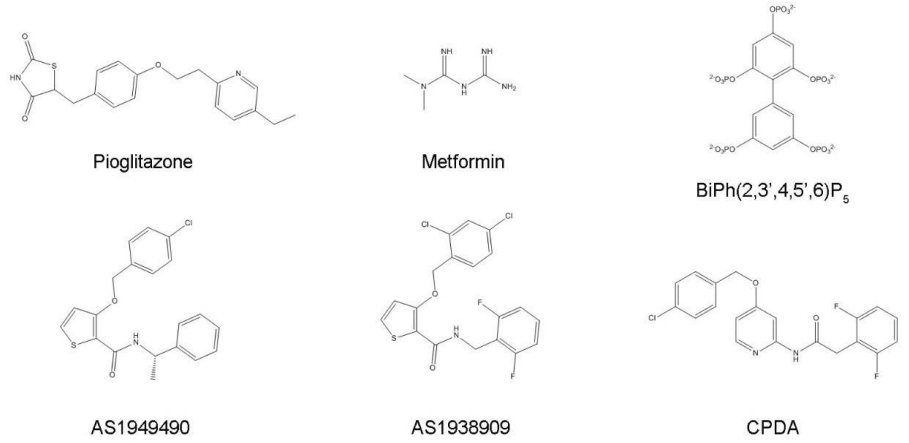

Figure 5. Structures of different insulin sensitizing drugs and small molecules that inhibit SHIP2.

3 AIMS OF THE STUDY

The mechanisms leading to the development of insulin resistance of podocytes and DN are not fully understood. Podocytes are insulin sensitive and translocate glucose transporters to the PM in response to insulin. Furthermore, insulin signalling is essential for normal kidney function and protection of the podocytes from apoptosis.

As defective insulin signalling and glucose transporter trafficking may lead to the development of insulin resistance, understanding the mechanisms behind these defects can lead to finding a cure for T2DM and potentially DN. This thesis sought to characterize the mechanisms involved in insulin responsive glucose transporter trafficking and regulation of podocyte apoptosis, and to find novel insulin sensitizers to fight DN by studying CD2AP and SHIP2.

CD2AP has been reported to interact with several proteins responsible for insulin signalling and vesicular trafficking, but its physiological role in these processes has not been studied. The expression of SHIP2, an interaction partner of CD2AP, is increased in the kidneys of db/db mice. Furthermore, lack of CD2AP and overexpression of SHIP2 increase the rate of apoptosis in podocytes, but their interplay in the regulation of podocyte apoptosis is elusive. SHIP2 is considered an excellent target to treat insulin resistance, yet all the known SHIP2 inhibitors are poor drug candidates. Identification of novel SHIP2 inhibitors could thus provide new drug leads for developing novel insulin sensitizers directly targeting the canonical insulin signalling pathway.

Specific aims of this thesis work were the following:

I. To characterize the role of CD2AP in the insulin signalling and glucose transporter trafficking.

II. To investigate the role of CD2AP and its interaction partner SHIP2 in podocyte apoptosis.

III. To identify novel SHIP2 inhibitors and study the effects of SHIP2 inhibition *in vitro* and *in vivo*.

4 MATERIALS AND METHODS

4.1 Animal and human studies

4.1.1 Experimental rodent models (Study II and III)

Female ICR mice and male db/db mice in C57BLKS background (BKS.Cg-m+/+Leprdb/BomTac, Scanbur, Karlslunde, Denmark) were used to test the effects of the potential SHIP2 inhibitors. Mice were treated with or without 250 mg/kg of metformin mixed to drinking water for 12 days, and received additional 250mg/kg dose of metformin per orally 4 h prior to sacrifice. CD2AP-/- and WT mouse kidneys embedded in OCT were a kind gift from the laboratory of Dr. Andrey Shaw at Washington University (St. Louis, MO), and were used to immunohistochemically detect ROS levels in the glomeruli and kidney cortex. Young male Sprague-Dawley rats were used to determine the effects of PA treatment on the protein levels of CD2AP and SHIP2 in the glomeruli. Nephrosis was induced by injecting intraperitoneally 150mg/kg of PA and sacrificed 10 days after the treatment. The National Animal Experiment Board in Finland approved the protocols, and all animal experiments were performed according to the approved guidelines.

4.1.1.1 Metabolic and renal functional assays (Study III)

Blood samples for glucose measurements were obtained from db/db mice treated with or without metformin by needle puncture of tail vein. 6 h fasting blood glucose concentrations were determined with Bayer's Elite Glucometer before initiation of metformin treatment and at the end of the experiment. Insulin tolerance test was performed after 12-day metformin treatment. Human regular insulin (0.75 U/kg) (Actrapid, Novo Nordisk, Denmark) was injected intraperitoneally to mice deprived of food for 6 h. Blood samples were collected before and 30, 60, 90, and 120 min after insulin administration. 24 h urine was collected individually in metabolic cages at the beginning and end of metformin treatment. Total urinary albumin content was measured at the Biochemical Analysis Core for Experimental Research (University of Helsinki) with ADVIA 1650 Chemistry System (Siemens AG Healthcare, Erlangen, Germany).

4.1.2 Morpholino antisense oligonucleotide injections in zebrafish (Study I)

Zebrafish embryos were staged according to somite number or hours or days post-fertilization (Kimmel, 1995). Four nanograms of translation blocking morpholino antisense oligonucleotide (MO) against CD2APL, and standard control MO (Gene Tools LLC, Philomath, OR), were injected into fertilized eggs using a PLI-90 microinjector (Harvard Apparatus, Cambridge, MA).

4.1.3 *In vivo* 2-NBDG uptake assay in zebrafish (Study I)

24-hpf morphants were injected in the yolk with 5 mg/ml 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG), a fluorescent glucose analogue (Molecular Probes, Inc., Eugene, OR) (Jensen *et al.*, 2010). After 10 min, the embryos were fixed with 4% PFA and imaged with a Zeiss Axioplan2 microscope. The mean fluorescence intensity of the embryos, excluding the yolk, was measured with constant settings using Image J 1.43u software.

4.1.4 Human study subjects (Study III)

Kidney samples were obtained from non-malignant parts of surgical nephrectomies of renal cancer patients with or without T2DM operated in Helsinki and Uusimaa Hospital district. The use of human material was approved by the hospital district's Ethics Committee and written informed consent was received from participants prior to inclusion in the study.

4.2 Antibodies and chemicals

Table 2. *Primary antibodies*

Antibody	Description	Reference / supplier	Study
8-OHdG	mouse monoclonal	Santa Cruz Biotechnology (Dallas, TX)	II
β-actin	rabbit polyclonal	Abcam (Cambrige, UK)	I
β-actin	mouse monoclonal	Sigma-Aldrich (St. Louis, MO)	II, III
pan-AKT	mouse monoclonal	R&D Systems (Minneapolis, MN)	I-III
phosphorylated AKT (Thr308)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II

phosphorylated AKT (Ser473)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	I, III
AMPK	mouse monoclonal	Santa Cruz Biotechnology (Dallas, TX)	III
AMPK	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	III
phosphorylated AMPK (Thr172)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	III
annexin V-FITC labelled	mouse monoclonal	Beckman Coulter (Brea, CA)	II
phosphorylated AS160 (Thr642)	rabbit polyclonal	Millipore (Billerica, MA)	I
cleaved caspase-3	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II, III
CD2AP 1764	rabbit polyclonal	Lehtonen *et al.*, 2000	I
CD2AP 1774	rabbit polyclonal	Lehtonen *et al.*, 2000	II
CD2AP 209	rabbit polyclonal	Lehtonen *et al.*, 2008	I
CD2AP 211	rabbit polyclonal	Lehtonen *et al.*, 2008	I
CD2AP H-290	rabbit polyclonal	Santa Cruz Biotechnology, Inc. (Dallas, TX)	II
CDK2	mouse monoclonal	Santa Cruz Biotechnology, Inc. (Dallas, TX)	II
clathrin heavy-chain	mouse monoclonal	Thermo Fisher Scientific (Waltham, MA)	I
GGA2	rabbit polyclonal	Santa Cruz Biotechnology, Inc. (Dallas, TX)	I
GGA2	mouse monoclonal	Santa Cruz Biotechnology, Inc. (Dallas, TX)	I
Glut1	rabbit polyclonal	Millipore (Billerica, MA)	I, III
Glut2	rabbit polyclonal	Abcam (Cambridge, UK)	III
GLUT4	rabbit polyclonal	Millipore (Billerica, MA)	I, III
HA.11	mouse monoclonal	Covance (Princeton, NJ)	I, III
HA.11 Alexa Fluor® 594 labelled	mouse monoclonal	Covance (Princeton, NJ)	I

IRAP	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	I
p44/p42 MAPK (ERK)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II
phosphorylated p44/p42 MAPK (Thr202/Tyr204)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II
nephrin	guinea pig polyclonal	Sigma-Aldrich (St. Louis, MO)	II
PDK1	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II
phosphorylated PDK1 (Ser271)	rabbit polyclonal	Cell Signaling Technology (Danvers, MA)	II
SHIP2 I-20	goat polyclonal	Santa Cruz Biotechnology, Inc. (Dallas, TX)	I-III
SHIP2	rabbit monoclonal	Cell Signaling Technology (Danvers, MA)	II
sortilin	rabbit polyclonal	Abcam (Cambridge, UK)	I
α-tubulin	mouse monoclonal	Sigma-Aldrich (St. Louis, MO)	I, III
WT1	rabbit polyclonal	Santa Cruz Biotechnology (Dallas, TX)	III

Table 3. *Secondary antibodies*

Secondary antibody	Description	Reference / supplier	Study
AlexaFluor 488	donkey anti-guinea pig	Invitrogen (Carlsbad, CA)	II
AlexaFluor 555	goat anti-rabbit	Invitrogen (Carlsbad, CA)	I, II
AlexaFluor 555	goat anti-mouse	Invitrogen (Carlsbad, CA)	I, III
AlexaFluor 594	goat anti-mouse	Invitrogen (Carlsbad, CA)	I
AlexaFluor 680	donkey anti-rabbit	Invitrogen (Carlsbad, CA)	I-III
AlexaFluor 680	donkey anti-mouse	Invitrogen (Carlsbad, CA)	I-III
IRDye 800	donkey anti-goat	LI-COR (Lincoln, NE)	II, III

IRDye 800	donkey anti-rabbit	LI-COR (Lincoln, NE)	I, II
IRDye 800	donkey anti-mouse	LI-COR (Lincoln, NE)	I-III
Trueblot®	HRP-conjugated anti-rabbit	Rockland Immunochemicals (Gilbertsville, PA)	I
DRAQ-5	nuclear dye (IR)	Thermo Fisher Scientific (Waltham, MA)	I, II
Hoechst	nuclear dye	Sigma-Aldrich (St. Louis, MO)	I-III

4.2.1 Chemicals (Study II and III)

Metformin (1,1-dimethylbiguanide) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except those noted below which were obtained from indicated suppliers. AS1949490 was synthesized by Dr. K. Wähälä and M. Berg (Department of Chemistry, University of Helsinki, Finland) or purchased from Tocris Bioscience (Bristol, UK).

4.3 Cell culture experiments

4.3.1 Cell culture (Study I-III)

Conditionally immortalized mouse CD2AP-/- and WT podocytes, a kind gift from Dr. Andrey Shaw (Washington University, St. Louis, MO), were maintained in high-glucose DMEM medium supplemented with 10% foetal bovine serum (FBS), ultraglutamine, penicillin, streptomycin and 10 U/ml interferon-gamma in 5% CO2 at +33°C (Schiffer *et al.*, 2004). L6 rat myoblast cells (CRL-1458; ATCC, Manassa, VA) were maintained in the same medium as described above but without interferon-gamma and in 5% CO2 at +37°C. To induce differentiation of L6 myoblasts to myotubes, cells were cultured 7-10 days in normal medium but supplemented with only 2% FBS. Conditionally immortalized human podocytes (AB 8/13) were cultured under standard conditions (Saleem *et al.*, 2002). Shortly, cells were maintained in permissive conditions in 5% CO2 at +33°C in RPMI media supplemented with 10% FBS, 1% glutamine, and insulin, transferrin and sodium selenite (ITS). To induce differentiation, podocytes were transferred to non-permissive conditions in 5% CO2 at +37°C for 14 days. Human embryonic kidney HEK293FT cells (Invitrogen, Carlsbad, CA) were maintained in DMEM supplemented with 10% FBS, ultraglutamine, penicillin and streptomycin in 5% CO2 at +37°C. Rat hepatoma FAO cells were maintained in Coon's modified Ham's F12 medium supplemented with 10% FBS, ultraglutamine, penicillin and streptomycin in

5% CO_2 at +37°C. All cell culture reagents were from Sigma-Aldrich, except ultraglutamine was from Lonza (Basel, Switzerland).

4.3.2 siRNA treatments of L6 myoblasts (Study I)

L6 myoblasts were transfected with 100 nmol ON-TARGET plus SMARTpool rat Cd2ap siRNA (L-098575-02), or siCONTROL Non-Targeting Pool#2 siRNA (D-001206-14-05) (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Thermo Fisher Scientific). After 4 h incubation with siRNA containing medium, cells were changed back to the culturing medium. Cells were used for experiments 72 h post transfection.

4.3.3 Transfections and infections (Study I-III)

CD2AP, HA-GLUT4-GFP and SHIP2 cDNAs were cloned into a lentiviral pSIN18.cppt.hEF1αp.WPRE vector (Gropp *et al.*, 2003) and empty pSIN18.cppt.hEF1αp.WPRE vector was used as control. Lentiviral human pLKO1-shSHIP2 vector shSHIP2 (Functional Genomics Unit, University of Helsinki, Finland) was used to knockdown SHIP2 in L6 myotubes and empty pLKO1 vector was used as control. HEK293FT cells were co-transfected with hEF1 carrying CD2AP, HA-GLUT4-GFP or SHIP2, or with pLKO1 carrying shSHIP2, and packaging plasmids KS pCMVΔ8.9 or pHCMV, respectively, using Lipofectamine 2000 (Invitrogen). Media were collected after 3 days, filtrated through a 0.45 μm filter and ultracentrifuged at 85,000 × g at +4°C for 90 min to concentrate the viruses.

4.3.4 Production of podocytes and L6 myoblasts stably overexpressing HA-GLUT4-GFP (Study I and III)

CD2AP-/- and WT podocytes, and L6 myoblasts were infected with lentiviruses carrying HA-GLUT4-GFP construct. Cells expressing HA-GLUT4-GFP were selected with puromycin treatment for 14 days, and the cells were sorted by fluorescence-activated cell sorting (FACS) based on GFP fluorescence and the populations of cell expressing medium level of HA-GLUT4-GFP were selected to be used in the following experiments.

4.4 Immunological methods

4.4.1 Immunoblotting (Study I-III)

Cells and tissues were lysed with 1% NP-40 buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) supplemented with 1× CompleteTM

proteinase inhibitor cocktail (Roche), 50 mM NaF and 1 mM Na3VO4) and rotated in cold. Detergent insoluble material was removed by centrifugation (16,000×g at +4°C for 10 min). Proteins were separated by SDS-PAGE and transferred to PVDF-FL membranes (Millipore), blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl), in Odyssei blocking buffer (LI-COR) or in Blocking buffer for fluorescent Western blotting (Rockland). Primary and secondary antibodies were diluted either in 1% skim milk in TBS or in commercial blocking buffer diluted 1/1 in TBS. Detection and quantification were performed with an Odyssey Infrared Imager (Li-COR Biotechnology) or by enhanced chemiluminescence using SuperSignal West Pico reagent (Thermo Fisher Scientific).

4.4.2 Immunofluorescence (Study I-III)

CD2AP-/- and WT podocytes were stimulated with 20 nM insulin as described above, fixed with 2% PFA, permeabilized with 0.1% Triton X-100 and blocked with CAS-block (Thermo Fisher Scientific). Rat kidneys were snap-frozen and embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands). Rat kidney cryosections were fixed in 3.5% paraformaldehyde (PFA), followed by incubation in 0.1% Triton X-100 in PBS at RT for 15 min and thereafter in 10% FBS or normal rat serum (NRS) at RT for 30 min. Samples were incubated with primary antibodies diluted in ChemMate (Dako) for 1 h followed by secondary antibodies diluted in ChemMate for 1 h. Samples for light microscopy were mounted in Moviol and examined with a Zeiss Axiophot 2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Samples for confocal microscopy were mounted in Vectashield (Vector, Burlingame, CA) and examined with a Leica TCS CARS SP8 confocal microscope.

4.4.3 In/On-Cell Western (Study I and II)

CD2AP-/- and WT podocytes, and L6 myotubes were grown on 96-well plates and fixed with 2% paraformaldehyde (PFA). Permeabilization with 0,1% TritonX-100 was done only to cells used for In-Cell Western. Cells were incubated with a primary antibody diluted in Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h followed by IRDye 800 donkey anti-mouse, or donkey anti-goat IgG and nuclear marker DRAQ-5. Detection and quantitation were performed with Odyssey Infrared Imager (LI-COR Biosciences).

4.4.4 Immunohistochemistry (Study II and III)

Human kidney samples were fixed with formaldehyde and embedded in paraffin. Immunoperoxidase stainings with antibodies directed against SHIP2

or WT1 were performed with a VectaStain Elite kit (Vector laboratories, Burlingame, CA) and AEC (Dako Cytomation). Slides were counterstained with hematoxylin and digitally scanned with 3DHistech Panoramic 250 FLASH II. Quantification of the staining intensity of SHIP2 was performed with HistoQuant module (3DHISTECH Ltd., Budapest, Hungary). Podocyte number was estimated by counting the number of WT1-positive cells per glomerular cross section in ten selected glomeruli per patient as previously described (Guo *et al.* 2012). For podocyte counting, only glomeruli larger than 170 μm in diameter were examined to ensure sectioning through the center of each glomerulus.

4.4.5 Immunoprecipitation (Study I-III)

Lysates, prepared as described in section Immunoblotting, were precleared with TrueBlot anti-rabbit Ig IP Beads (eBioscience, San Diego, CA) or protein G sepharose beads (Thermo Fisher Scientific). For immunoprecipitation of the proteins, lysates were incubated with 5 μl of serum-based antibodies or 5 μg of purified commercial antibodies overnight. For negative controls, the lysates were incubated with 5 μl normal serum or 5 μg purified IgG derived from the same animal species as the primary antibody was derived from. Immune complexes were collected by binding them to sepharose beads for 1-2 h and immunoblotted as described above. All the steps were performed at +4°C.

4.4.6 *In situ* proximity ligation assay (Study I)

CD2AP-/- and WT podocytes were fixed with 2% PFA and blocking and primary antibodies were applied as described above in Immunofluorescence. Duolink *in situ* proximity ligation assay was carried out according to the manufacturer's instructions (Olink Biosciences, Sigma-Aldrich). Imaging was performed with a Zeiss Axiophot 2 microscope and quantification with the Duolink ImageTool software (Olink Biosciences).

4.5 Molecular biology and biochemical methods

4.5.1 Cloning, expression and purification of recombinant phosphatases (Study III)

For the biological testing, we produced the phosphatase domains of human SHIP2 and human SHIP1 (Suwa *et al.* 2009). The DNAs of the phosphatase domains were inserted into pRSETA-vector possessing an N-terminal 6xHis tag and transformed to E. coli.

Production of 6xHis-SHIP2 and 6xHis-SHIP1 phosphatase domains was induced by IPTG induction. After overnight culture, bacteria were pelleted and suspended to lysis buffer (TBS, 10 mM inositol, Roche Complete protease inhibitor cocktail). Bacteria suspension was sonicated and the cell debris was removed by centrifugation 13600 x g for 20 min.
His-tagged SHIP2 and SHIP1 were purified by binding to Ni2+-sepharose beads for 1 h. His-tagged proteins were eluted with elution buffer (TBS, 250 mM inositol) and inositol was removed by dialysis. All the purification steps were performed in +4°C using ice cold solutions.

4.5.2 Quantitative RT-PCR (qRT-PCR) (Study III)

Total RNA was isolated from mouse liver tissue using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) as described previously (Dash *et al.* 2014). Quantitative RT-PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laborotories, Hercules, CA).

4.5.3 Sub-cellular fractionation (Study I)

CD2AP-/- and WT podocytes were homogenized with ice-cold HES buffer (20 mM Hepes pH 7.4, 1 mM EDTA, 225 mM sucrose) by pulling through 25g and 26g needles. Nuclei were removed by centrifugation (1,000 x g for 5 min). Membrane and cytosolic fractions were separated by centrifugation of the post-nuclear supernatant (PNS) at 19,000 × g for 22 min. The cytosolic fraction was centrifuged at 41,000 × g for 22 min to pellet the high-density microsomal fraction (HDM). The obtained supernatant was centrifuged at 180,000 × g for 77 min to pellet the low-density microsomal fraction (LDM). All centrifugation steps were performed at +4°C. The HDM and LDM pellets were re-suspended in HES buffer and analysed by immunoblotting as described below.

4.5.4 16,000×g fractionation (Study I)

CD2AP-/- and WT podocytes were homogenized as described above. Nuclei were removed by centrifugation (500 × g at +4°C for 10 min). Donor membranes and the GSV vesicle fraction were separated by centrifugation at 16,000 × g at +4°C for 20 min. Supernatant was collected into a separate tube and the pellet was re-suspended in PBS. The volume of the pellet fraction loaded to the gel was 5 times the volume of the supernatant.

4.6 Functional assays

4.6.1 H^3 2-DOG uptake (Study I and III)

CD2AP-/- and WT podocytes, and HA-GLUT4-GFP expressing L6 myotubes were grown in 12-well plates until they were 70% confluent or fully differentiated and then serum starved overnight. Cells were incubated in Krebs-Ringer buffer (128mM NaCl, 5,2mM KCl, 1,4mM CaCl2, 1,4mM MgSO4, 10mM Na2HPO4) or glucose free DMEM containing 20-200 nM insulin followed by addition of 1 μCi/ml 2-deoxy-D-[(1, 2-3H(N)]-glucose (PerkinElmer, Waltham, MA). Glucose uptake was stopped by transferring plates on ice and cells were lysed using 1% TritonX-100 in PBS (10 mM phosphate pH 7.4, 137 mM NaCl). One ml of scintillation fluid was added on lysates containing 10-20 μg of protein. Glucose taken up by the cells was measured using Wallac 1450 MecroBeta TriLux Liquid Scintillation Counter (Perkin Elmer-Cetus, Waltham, MA).

4.6.2 HA-GLUT4-GFP uptake assay (Study I)

CD2AP-/- and WT podocytes and L6 myotubes, all stably expressing medium levels of HA-GLUT4-GFP, were cultured as for On-Cell Western. HA antibody was diluted 1/100 in serum-free medium with or without 20 mM insulin and incubated with the antibody-containing medium for 30, 15 or 5 min. The plate was transferred on ice and the antibody-containing medium was added to the 0 min wells to label HA on the plasma membrane. Rest of the protocol was performed as described above (In/On-Cell Western).

4.6.3 HA-GLUT4-GFP endocytosis assay (Study III)

L6 cells expressing HA-GLUT4-GPF were cultured and treatments performed similarly to On-Cell Western. Cells were transferred on ice, incubated with HA antibody in serum free medium for 15 min followed by additional incubation in fresh 2% FBS containing culture medium for 15 min, and fixed with 2% PFA for 20 min. Detection was done as described in In/On Cell Western.

4.6.4 Live cell imaging (Study I)

CD2AP-/- and WT podocytes stably expressing HA-GLUT4-GFP were cultured on Chamber Slides (Thermo Fisher). Cells were treated with fluorescently labelled HA antibody diluted 1/100 for 20 min. After 30 min of imaging, the cells were stimulated with 20 nM insulin and imaging was continued for one hour. Time-lapse video imaging was performed with a Leica

TCS CARS SP8 confocal microscope (Wetzlar, Germany). 3D-rendering and time-lapse videos were done using Imaris 7.5 software (Bitplane Scientific Software, Zurich, Switzerland).

4.6.5 Induction and detection of apoptosis (Study II and III)

Differentiated human podocytes were exposed to PA (Sigma-Aldrich, 50 μg/ml) for 48 h. AS1949490, a small molecule inhibitor of SHIP2 (Suwa et al,. 2009), was or was not added 24 h after starting the PA treatment. CD2AP-/- and WT podocytes were treated or not for 24 h with AS1949490 prior to detection. Apoptosis was detected by flow cytomerty using Annexin V-FITC and 7-AAD double staining with FACSAria (BD Biosciences, Franklin Lakes, NJ) or CyAn ADP (Beckman Coulter, Brea, CA). Cells positive for AnnexinV-FITC and negative for 7-AAD were deemed apoptotic. A total 1 x 104 cells were detected by FACS in each sample.

4.6.6 Measurement of ROS production (Study II)

CD2AP knockout podocytes were grown in 96 well plates and treated with 10 μM AS1949490 for 48 h. ROS was measured using 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (Sigma). Cells cultured on 96-well plates were incubated with 50 μM DCFH-DA fluorescent probe and 2 μM Hoechst 33342 (Fluka, Sigma Aldrich) for 60 min. Fluorescence intensity was measured at 485 nm excitation wavelength and 525 nm emission wavelength using a Fluoroskan Ascent FL luminometer (Thermo Fisher Scientific).

4.6.7 Malachite green phosphate assay (Study II and III)

The catalytic activity of purified His-tagged SHIP2 and SHIP1 phosphatase domain, purified PTEN and SHIP2 immunoprecipitated from various cell types and tissues was determined using D-myo-phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) (Echelon). 100 ng of purified His-tagged SHIP2 phosphatase domain, 100ng of purified PTEN or 10μl of immunoprecipitation beads were let to react with 100μM PtdIns(3,4,5)P3 in enzyme reaction buffer (10 mM HEPES pH 7.4, 6 mM MgCl2, 0.1% CHAPS, 250 mM sucrose, and 0.25 mM EDTA)with or without inhibitors for 20 min. Biomol green reagent (Enzo Life sciences) was added (to each well), and the plate was incubated for another 25 min. Absorbance at 620 nm was measured using FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany).

4.7 Computational methods

4.7.1 Computationally determining the location of the active site of SHIP2 from the protein structure (Study III)

For in silico studies we used two SHIP2 structures. One was obtained from protein data bank (PDB) and the other from SWISS-MODEL Repository. To determine the active site of SHIP2 we probed all possible binding cavities with a model of PI(3,4,5)P3 ring structure and the known SHIP2 inhibitor AS1949490. PI(3,4,5)P3 and AS1949490 were docked to the cavities on the protein structure using CDOCKER with CHARMm force field. We found only one cavity that bound both PI(3,4,5)P3 and AS1949490 and was found in both the crystal structure and the computational model of SHIP2.

4.7.2 Virtual screening (Study III)

The High-throughput Center Helsinki libraries of FDA approved drugs, Spectrum2009, Tripos structures, ChemBridge CNS Set, ChemBridge DiverSet, ChemDiv 25000 and ChemDiv 25040, a total of 88640 molecules, were used for the virtual screening. Conformational libraries of these libraries were created with Discovery studio 4.0 (Accelrys, San Diego, CA). CHARMm force field and MMFF94 charges were calculated for all the molecules. CDOCKER was used to dock the ligands to the active site of both the PDB and SWISS-MODEL structure. All duplicates were removed and only the conformation having the highest docking interaction energy was saved. For biological testing we selected molecules with docking interaction energy less than the average interaction energy of 10 best conformations of AS1949490 . This resulted in 379 compounds to be tested biologically. After the biological testing, 10 pan-assay interference compounds (PAINS) (Baell *et al.* 2010) and 7 compounds that failed the turbidity test were discarded from the final results.

4.8 Statistical methods (Study I-III)

All variables are presented as mean ±SD or ±SEM. The significance of differences between groups was determined by one-way ANOVA or Students t-test (GraphPadPrism6, GraphPad Software Inc, La Jolla, CA; Microsoft Office, Microsoft Inc, Redmond, WA), and p-values of less than 0.05 were considered statistically significant.

5 RESULTS

5.1 Study I: Lack of CD2AP disrupts GLUT4 trafficking and attenuates glucose uptake in podocytes

As CD2AP is known to interact with proteins involved in vesicle trafficking (Havrylov *et al.*, 2008; Wasik *et al.*, 2012) and insulin signalling (Huber *et al.*, 2003; Schiffer *et al.*, 2004; Kobayashi *et al.*, 2004 Hyvönen 2010), we studied the role of CD2AP in glucose uptake and glucose transporter trafficking in basal state and after insulin stimulation in CD2AP-/- and WT podocytes and L6 myoblasts.

5.1.1 Absence of CD2AP reduces glucose uptake

We found that lack of CD2AP decreases the glucose uptake in the basal state by 30% in podocytes (Study I, Fig 1A). Interestingly, we also discovered that in L6 myoblasts 50% knockdown of CD2AP using siRNA was enough to decrease glucose uptake by 20% (Study I, Fig S1A-C). The glucose uptake in CD2AP-/- podocytes did not increase after 15 min treatment with insulin whereas in WT podocytes it increased by 19% (Study I, Fig 1B). We next investigated whether this results from a disruption in the insulin signalling cascade or decreased expression of glucose transporter molecules. We observed a dose-dependent increase in serine 473 phosphorylation of AKT and threonine 642 phosphorylation of AS160 in response to insulin in both CD2AP-/- and WT podocytes (Study I, Fig S2A-D). Quantification of glucose transporter 1 and 4 (Glut1 and 4) protein levels revealed that both cell types had similar Glut1 levels but CD2AP-/- podocytes had 20% higher GLUT4 protein levels compared to WT podocytes (Study I, Fig 1C and D). These data suggest that lack of CD2AP does not disrupt the activation of the insulin signalling pathway but that CD2AP might have a role in glucose transporter trafficking.

5.1.2 Trafficking of GLUT4 is disrupted in CD2AP-/- podocytes

We created WT and CD2AP-/- podocyte lines stably expressing HA-GLUT4-GFP to study the role of CD2AP in GLUT4 trafficking. The HA-tag is located in the extracellular domain of GLUT4 thus providing an excellent tool for distinguishing cytosolic GLUT4 from GLUT4 bound to the plasma membrane. By applying antibodies against HA-tag to unpermeabilized cells, we could stain only GLUT4 molecules bound to plasma membrane. We discovered that in CD2AP-/- podocytes HA-GLUT4-GFP did not translocate to the plasma

membrane in response to insulin, whereas in WT podocytes it did (Study I, Fig 2A-E). HA internalization assay was performed to investigate the rate of GLUT4 recycling between the plasma membrane and cytosol. We found the recycling rate to be significantly slower in CD2AP-/- podocytes compared to WT podocytes (Study I, Fig 2F). To further investigate this, we pulse-labelled the cell lines with fluorochrome-labelled HA antibody and followed its trafficking with 4D live cell microscopy in basal state and after insulin stimulation. In WT podocytes we could detect dynamic flux of GLUT4 in basal state, and the flux increased after insulin stimulation. In CD2AP-/- podocytes HA signal accumulated in cytosol in basal state, but in contrast to WT podocytes, it did not respond to insulin stimulation (Study I, Fig 3A, B and Supplemental movies 1-4). This indicates that GLUT4 trafficking is disrupted in the absence of CD2AP.

5.1.3 CD2AP co-localizes with GSV markers

While investigating the role of CD2AP in GLUT4 trafficking, we found it to locate in the same microsomal fractions as IRAP, GLUT4 and Sortilin in podocytes (Study I, Fig 4A). We further studied the insulin-induced translocation of these GSV markers in CD2AP-/- and WT podocytes using immunofluorescence microscopy. IRAP, a marker for insulin responsive GSVs (Waters *et al.* 1997), diffused from its perinuclear localization in response to insulin in WT but not in CD2AP-/- podocytes (Study I, Fig 4B). We did not detect a clear translocation of Sortilin, a crucial component for the formation of insulin responsive GSVs (Shi and Kandror, 2005), in response to insulin in either cell type, but observed that its protein level was 3-fold higher in CD2AP-/- podocytes (Study I, Fig 4C and D). These data imply that the trafficking of insulin-responsive GSVs is impaired in the absence of CD2AP.

5.1.4 CD2AP interacts with components of GSVs and the GLUT4 trafficking machinery

As CD2AP was detected in the same microsomal fractions as GSV markers, we hypothesised that CD2AP could interact with these proteins. To study this, we performed co-immunoprecipitations on lysates prepared from WT podocytes. We were not able to detect an interaction between CD2AP and Sortilin even though we used several antibodies targeting different epitopes of CD2AP. Therefore, we moved on to investigate the only protein known to bind to the cytosolic domain of Sortilin – GGA2 (Nielsen *et al.* 2001). Co-immunoprecipitation revealed that CD2AP interacts specifically with GGA2 of the GGA protein family (Study I, Fig 5A and S3). To confirm this new interaction with another technique and to quantify whether insulin induces changes in this interaction, we used proximity ligation assay (PLA). Indeed,

PLA confirmed that CD2AP and GGA2 interact, and revealed that insulin stimulation enhanced the interaction in WT podocytes (Study I, Fig 5B-F). As GGA2 is also known to be a clathrin adaptor (Puertollano *et al.* 2001), we investigated whether lack of CD2AP affects the interaction between GGA2 and clathrin under serum starved or insulin-stimulated conditions. Absence of CD2AP increased the interaction between GGA2 and clathrin by about 30%, and insulin treatment further increased the interaction by 25% (Study I, Fig 6A). In WT podocytes we did not detect an increase in GGA2-clathrin interaction but the localization of the PLA signal changed from perinuclear to more diffuse after insulin stimulation (Study I, Fig 6B and C). On the contrary, in CD2AP-/- podocytes the PLA signal condensed to the perinuclear region after insulin stimulation (Study I, Fig 6D and E). Interestingly, CD2AP is reported to harbour a clathrin-binding FxDxF motif (Brett *et al.* 2002) but the interaction has not been biologically confirmed. Indeed, we observed that CD2AP forms a complex with clathrin and that the interaction is negatively regulated by insulin (Study I, Fig 7A-C).

5.1.5 CD2AP links clathrin-coated vesicles to actin

To determine whether CD2AP affects the budding of GLUT4-containing vesicles from the donor membranes located in the TGN, we separated GSVs from the donor membranes by differential centrifugation. Lack of CD2AP did not alter the ratio of GLUT4 between the donor membranes and GSVs in basal or insulin-stimulated conditions (Study I, Fig 7D and E). Next, we investigated whether CD2AP acts as a linker between clathrin and actin, as it is known to regulate the assembly of actin on vesicles (Carreno *et al.*, 2004). Absence of CD2AP decreased actin-clathrin interaction by 38% (Study I, Fig 7F-J). Insulin stimulation decreased the interaction by 24%, but only in WT podocytes that express CD2AP (Study I, Fig 7F-J). Probing clathrin from the GSV and donor membrane fractions before and after insulin stimulation suggested that the recycling of free clathrin back to donor membranes is defective in the absence of CD2AP (Study I, Fig 7K and L).

5.1.6 Knockdown of CD2AP attenuates glucose uptake in zebrafish *in vivo*

To verify that our findings are not just an anomaly occurring in cultured cells but are applicable to whole organisms, we knocked down *CD2APL,* a zebrafish orthologue of human *CD2AP*, in zebrafish. This caused pericardial and yolk sac oedema as expected (Study I, Fig 8A and B) (Hentschel *et al.*, 2007). Knockdown of *CD2APL* also led to 42% reduction in glucose uptake, verifying that CD2AP has a significant role in glucose homeostasis *in vivo*.

5.2 Study II: Inhibition of SHIP2 in CD2AP-deficient podocytes ameliorates reactive oxygen species generation but aggravates apoptosis

Excessive ROS production and lack of CD2AP are separately known to result to podocyte injury and apoptosis (Susztak *et al.*, 2006; Schiffer *et al.*, 2004). Also, the overexpression of SHIP2, an interaction partner of CD2AP, induces podocyte apoptosis *in vitro* (Hyvönen *et al.*, 2010).

5.2.1 Lack of CD2AP increases ROS production and apoptosis in mouse podocytes *in vitro* and *in vivo*

To study whether CD2AP has a role in oxidative stress and the consequent apoptosis, we analysed ROS production using DCFH-DA fluorescent probe assay and apoptosis by FACS with annexin V and 7-AAD labelling in CD2AP-deficient and WT podocytes. Absence of CD2AP increased ROS production by 25% and apoptosis by 2,8-fold compared to WT podocytes (Study II, Fig 1A and B). Interestingly, we could also detect increased ROS in kidney sections of 3-week-old CD2AP-/- mice (Study II, Fig 4). Transfecting CD2AP back to CD2AP knockout podocytes restored the rate of apoptosis back to the same level as observed in WT podocytes, despite the low expression level of reintroduced CD2AP (Study II, Fig 1B and C). As phosphorylation of proteins in survival signalling pathways, such as AKT and ERK, are vital for the activation of the pathways, we studied whether the phosphorylation of the these proteins is reduced in the CD2AP-/- podocytes. In the absence of CD2AP, the phosphorylation of T308 of AKT reduced by 30%, but the phosphorylation of S473 of AKT remained unchanged (Study II, Fig 1D-F). The phosphorylation of ERK was 40% lower in CD2AP-/- podocytes compared to WT podocytes. The data reveal that ROS production is increased and the activity of pro-survival pathways are attenuated in the absence of CD2AP.

5.2.2 Inhibition of SHIP2 activity in the absence of CD2AP decreases ROS production

Next, we investigated whether CD2AP and SHIP2, known interaction partners (Hyvönen *et al.* 2010), have a functional interrelation. The protein level of SHIP2 was increased by 30% and the phosphatase activity by 26% in the absence of CD2AP (Study II, Fig 2A-C). Treating the CD2AP-/- and WT podocytes with a known SHIP2 inhibitor AS1949490 (Suwa *et al.* 2009) decreased the activity of SHIP2 by 33% compared to WT podocytes and by 47% compared to untreated CD2AP-/- podocytes (Study II, Fig 2C). Inhibition of SHIP2 activity in CD2AP-/- podocytes reduced the production of ROS to the

same level as observed in WT podocytes (Study II, Fig 2E). Surprisingly, this did not reduce the level of apoptosis but rather increased it by 40% (Study II, Fig 2F).

To elucidate the mechanisms behind the increased apoptosis in CD2AP-/- podocytes, we investigated whether the protein levels of two anti-apoptotic proteins, PDK1 and CDK2, are decreased when CD2AP is absent. Indeed, PDK1 level was 35% and CDK2 level 65% lower in CD2AP-/- podocytes compared to WT podocytes (Study II, Fig 2 G and H). Treatment with AS1949490 further decreased the amount of PDK1 but only in CD2AP-/- podocytes (Study II, Fig 2I). To investigate the effect of AS1949490 on the signalling pathways associated with increased apoptosis, we studied the activation of AKT, ERK and PDK1 in response to AS1949490 treatment in the presence and absence of CD2AP. Regardless of the expression of CD2AP, treatment with AS1949490 increased the T308 phosphorylation of AKT (Study II, Fig 3A and B). Interestingly, the treatment increased S473 phosphorylation of AKT only in the presence of CD2AP (Study II, Fig 3C). We detected also increase in the activation of PDK1 in CD2AP-/- and WT podocytes in response to AS1949490 treatment (Study II, Fig 3D and E). AS1949490 increased the phosphorylation of ERK in both cell types, but significantly more in WT podocytes compared to CD2AP-/- podocytes (Study II, Fig 3F). Together these data suggest that interplay between SHIP2 and CD2AP is necessary for cell survival and for full activation of AKT in response to AS1949490 treatment.

5.2.3 PA-treatment of cultured human podocytes downregulates CD2AP, upregulates SHIP2 and increases ROS production and apoptosis

Puromycin aminonucleoside (PA) has been reported to induce DNA damage *via* ROS in podocytes (Marshall *et al.*, 2006) and to induce apoptosis in a dose- and time-dependent manner (Sanwal *et al.*, 2001). To analyse the effects that PA might have on CD2AP and SHIP2 *via* the increased ROS, we treated cultured human podocytes or rats with PA. The PA-treatment of human podocytes decreased the expression of CD2AP by 80% and increased the expression of SHIP2 by 34% and its phosphatase activity by 45% (Study II, Fig 5A-D). Immunofluorescence staining of the kidneys of rats with PA-induced nephrosis also revealed that the expression level of CD2AP appeared diminished and the expression level of SHIP2 increased when compared to the non-treated littermates (Study II, Fig 8). ROS generation was measured from PA-treated cultured human podocytes, and was increased by 60%, and the apoptosis increased 5-fold compared to untreated podocytes (Study II, Fig 5E and F) confirming previous findings (Sanwal *et al.*, 2001).

5.2.4 SHIP2 inhibition alone does not reduce ROS production or apoptosis in human podocytes treated with PA

As SHIP2 activity was increased after PA treatment in human podocytes, we tested whether administering AS1949490 reduces the production of ROS and potentially also ameliorates apoptosis. First, we confirmed that AS1949490 treatment indeed prevents the increase in the phosphatase activity of SHIP2 caused by PA-treatment (Study II, Fig 6A). However, we could not detect any decrease in ROS production or in the rate of apoptosis comparing podocytes treated with PA alone or with PA together with AS1949490 (Study II, Fig 6B and C). This indicates that the inhibition of SHIP2 activity alone is not enough to rescue the podocytes from the immense damage caused by PA.

5.2.5 Overexpression of CD2AP in human podocytes reduces ROS production and protects from apoptosis induced by PA

Treatment of human podocytes with PA has been previously reported to downregulate PDK1 and CDK2 (Saurus *et al.* 2016). To define whether overexpression of CD2AP attenuates the effects of PA, we overexpressed CD2AP in human podocytes concurrently with PA-treatment. Indeed, overexpression of CD2AP restored the levels of PDK1 and CDK2 back to the levels observed in non-treated podocytes (Study II, Fig7 A and B). The overexpression of CD2AP alone did not have an effect on the protein levels of PDK1 and CDK2 (Study II, Fig 7A and B). CD2AP overexpression also reduced the PA-induced production of ROS (Study II, Fig 7C), and rescued the podocytes from PA-induced apoptosis restoring the rate of apoptosis back to the same level as in untreated podocytes (Study II, Fig 7D). The overexpression of CD2AP alone did not affect the production of ROS or the level of apoptosis in podocytes (Study II, Fig 7C and D).

5.3 Study III: Metformin enhances insulin sensitivity and protects against kidney injury by inhibiting SHIP2 activity

As SHIP2 negatively regulates insulin signalling, it is a good target for insulin sensitizing drugs. The currently known SHIP2 inhibitors, such as AS1949490, have been identified by biological high-throughput screening and have poor bioavailability. To identify novel SHIP2 inhibitors with better drug-like properties, we conducted a structure-based virtual screening, and validated one of the top hits, metformin, as a novel SHIP2 inhibitor both *in vitro* and *in vivo*.

5.3.1 Screening for novel SHIP2 inhibitors

To locate the active site of SHIP2 we used the crystal structure 3NR8 and the computational structure model O15357 of the phosphatase domain of human SHIP2 protein. We identified only one homologous cavity from both structures that bound AS1949490 and PI(3,4,5)P_3, and the site was identical with the one determined by Mills *et al.* (Mills *et al.*, 2012). Next, we docked the small molecular libraries to the active site of SHIP2 *in silico*. A total of 88 640 molecules and their different conformations were screened and of these, 2 636 molecules bound to the active site of SHIP2. Of these, 379 molecules had lower interaction energies than AS1949490 and were selected for biological validation. For this, we produced a His-tagged human SHIP2 phosphatase domain, and for specificity testing we generated a His-tagged human SHIP1 phosphatase domain in *E. coli*. The phosphatase activities were measured using malachite green phosphatase assay that detects free phosphate groups. We found 9 molecules that inhibited at least 50% and 26 molecules that inhibited at least 25% of SHIP2 activity with a concentration lower than 50 μM. Four of these molecules are used currently as drugs and had IC_{50} values lower than 10 μM (Study III, Table 1). One of these was the first line drug to treat type 2 diabetes – metformin (Study III, Fig 1A).

5.3.2 Metformin as a potent SHIP2 inhibitor

Biological testing revealed that metformin inhibits 39% of the phosphatase activity of recombinant SHIP2 with an IC_{50} value of 6,1 μM, but did not significantly inhibit SHIP1 or PTEN activity (Study III, Fig 1B and Table 1). To confirm that metformin inhibits full length SHIP2 in cultured cells, we treated L6 myotubes and podocytes with 1 mM metformin, immunoprecipitated SHIP2 from the cell lysates and measured its phosphatase activity. Metformin inhibited SHIP2 activity by 24% in myotubes and 14% in podocytes (Study III, Figure 1C and D). Interestingly, metformin did not inhibit SHIP2 in hepatocytes (Study III, Fig 1SA). As plasma concentration of metformin in patients is 8-30 μM (Liu *et al.*, 2009), we treated cells with 20 μM metformin or the known SHIP2 inhibitor AS1949490 for comparison. We observed 30% decrease in SHIP2 activity in myotubes and 20-30% decrease in podocytes, with no significant difference between metformin and AS1949490 in their ability to inhibit SHIP2 (Study III, Fig 1E and F). Analysis of the SHIP2 protein level in metformin-treated cells confirmed that the lowered activity was not caused by downregulation of SHIP2 (Study III, Fig 2SA-D, I and O). These results imply that metformin is a specific and as potent SHIP2 inhibitor as AS1949490 *in vitro*.

5.3.3 Inhibition of SHIP2 activity with metformin enhances glucose uptake into cells

To study the effect of metformin on glucose uptake and GLUT4 trafficking, we created an L6 cell line overexpressing HA-GLUT4-GFP (Study III, Fig S4A and B). Insulin treatment increased the glucose uptake of these cells by 20% and metformin by 215%, but metformin did not potentiate the effect of insulin (Study III, Fig 2A). Cultured human podocytes were unresponsive to insulin alone, but metformin alone increased their glucose uptake by 50% and together with insulin by 80% (Study III, Fig 2B). To confirm that the increased glucose uptake is due to inhibition of SHIP2 activity by metformin, we overexpressed SHIP2 in L6 and L6-GLUT4 myotubes (Figure S5C and D). The overexpression increased the activity of SHIP2 by 50% and metformin restored the activity to the same level as in untreated cells (Study III, Fig 2E). The increase in SHIP2 activity was not caused by higher amount of immunoprecipitated SHIP2 protein (Study III, S5E). SHIP2 overexpression also reduced glucose uptake by 20-25% and attenuated the effect of metformin to increase it in basal and insulin-stimulated states (Study III, Fig 2F and G). To confirm this, we knocked down SHIP2 in L6-GLUT4 cells using lentiviral shRNA, reaching 30-45% reduction in SHIP2 expression (Study III, S5A and B). This reduced metformin-stimulated glucose uptake by 16% compared to cells infected with the empty vector (Study III, Fig 2D). The data reveal that metformin increases the glucose uptake of the cells, but both the overexpression and the knockdown reduce the ability of metformin to increase glucose uptake.

5.3.4 AMPK activation does not induce glucose uptake in human podocytes

To define whether metformin enhances the insulin signalling pathway by inhibiting SHIP2, we quantified serine 473 phosphorylation of AKT, but did not detect any change in L6 myotubes or podocytes (Study III, Fig S3A, B, G and L). Metformin is known to activate AMPK (Zhou *et al.* 2001) and we confirmed this in L6 myotubes, podocytes and hepatocytes by quantifying the increase in the phosphorylation of AMPK (Study III, Fig S3A-C, H, M and P). The well-established AMPK activator AICAR enhances glucose uptake in L6 cells (Turban *et al.* 2012), but in contrast to this, we did not detect increased glucose uptake in human podocytes treated with AICAR alone or in combination with insulin (Study III, Fig 2C). Yet, AICAR activated AMPK in podocytes as expected (Study III, Fig S3B and N). This indicates that the activation of AMPK is only partially responsible for the ability of metformin to increase glucose uptake or that the effects are cell type-specific.

5.3.5 Metformin increases GLUT4 on plasma membrane by slowing down its endocytosis

Metformin has been reported to slow down glucose transporter endocytosis and thus to increase glucose uptake (Yang and Holman 2006). Using HA-GLUT4-GFP myocytes we confirmed that metformin increases the amount of GLUT4 on the plasma membrane by 40% and slows down the endocytosis of GLUT4 by 50% compared to cells at basal state or stimulated with insulin (Study III, Fig 3A and B). Metformin treatment did not have an effect to GLUT4 or GLUT1 protein levels either in L6 cells or podocytes (Study III, Fig S3A, B, E, F, J and K).

5.3.6 SHIP2 overexpression-induced apoptosis is prevented by metformin

We have previously shown that SHIP2 overexpression induces apoptosis in human podocytes (Hyvönen *et al.* 2010). Thus, here we tested whether metformin prevents this by inhibiting SHIP2 activity. We achieved 50% overexpression of SHIP2 that significantly increased the cleavage of caspase-3. Metformin restored the level of cleaved caspase-3 to the level observed in the control (Study III, Fig 4A and B). Overexpression of SHIP2 decreased the insulin-stimulated phosphorylation of S473 of AKT by 30% and metformin restored it back to the same level as in empty vector -infected podocytes (Study III, Fig 4D and E).

5.3.7 Metformin inhibits SHIP2 activity *in vivo*

To confirm that metformin inhibits SHIP2 *in vivo*, we treated normal ICR mice with 250mg/kg of metformin, resulting to the equivalent plasma concentration as detected in humans (Foretz *et al.*, 2014) for 10 days and immunoprecipitated SHIP2 from the skeletal muscle and kidney tissues to measure its activity. We found that metformin decreased the activity of SHIP2 by 20% in both tissue types (Study III, S6A and B). Metformin did not affect the body weight or fasting blood glucose (Study III, Fig S6C and D). Encouraged by the detected SHIP2 inhibition, we treated diabetic db/db mice with metformin for 12 days and found that SHIP2 activity decreased by 40% in skeletal muscle and kidney in response to the treatment (Study III, Fig 5A and B). Interestingly, we did not detect any decrease in SHIP2 activity in the liver of the metformin treated mice (Study III, Fig S2B). Parallel to what we observed in cultured cells, metformin did not change the expression level of SHIP2 *in vivo* (Study III, Fig S7A-D, I and N). Metformin did not activate signalling through AKT or AMPK, nor did it affect GLUT1 or GLUT4 protein levels (Study III, Fig S7A-C, E-H, J-M and O). Metformin treatment for 12 days did not affect body weight, urinary albumin excretion or fasting blood glucose

of db/db mice, in line with the results obtained from ICR mice (Study III, Fig 5C-E). To assess whether metformin has an effect on whole-body glucose metabolism, we performed insulin tolerance test and found that metformin-treated mice were insulin sensitive unlike the untreated db/db mice (Study III, Fig 5F and G). Metformin also reduced the mRNA levels of PCK1 and glucose 6-phosphatase in the liver (Study III, Fig S2C and D), as reported previously (Minassian *et al.* 1998 and Yuan *et al.* 2002). These data reveal that metformin inhibits SHIP2 and increases insulin sensitivity *in vivo*.

5.3.8 Metformin reduces the activity of SHIP2 and podocyte loss in kidneys of T2DM patients

As metformin is widely used for treatment of T2DM in humans and has been shown to reduce albuminuria (Amador-Licona *et al.*, 2000), we studied its effects on SHIP2 in kidneys of patients with T2DM (Study III, Table S2). Immunohistochemistry-based quantification of SHIP2 expression level in glomeruli and kidney cortex revealed no difference between patients with T2DM receiving metformin or non-metformin medication, nor between patients with T2DM and people without diabetes (Study III, Fig 6A-C). Yet, the SHIP2 activity was increased by 37% in the kidneys of patients with T2DM with non-metformin mediation compared to people without diabetes. The activity of SHIP2 in patients with T2DM receiving metformin did not significantly differ from people without diabetes (Study III, Fig 6D). As metformin decreased apoptosis caused by increased SHIP2 expression level and activity in cultured podocytes, we investigated whether metformin treatment associates with podocyte loss in T2DM patients. Indeed, we observed that patients with T2DM with non-metformin medication had 38% less podocytes in their glomeruli whereas patients receiving metformin had only 11% less podocytes compared to people without diabetes (Study III, Fig 7A and B). These data, together with the data on cultured cells, suggest that metformin prevents podocyte loss by apoptosis by inhibiting the catalytic activity of SHIP2.

6 DISCUSSION

6.1 Role of CD2AP in GLUT4 trafficking in podocytes

We observed that in podocytes lacking CD2AP, basal glucose uptake is attenuated and insulin-stimulated glucose uptake is abrogated. This was not due to decreased expression of glucose transporters, as absence of CD2AP did not affect the expression of GLUT1, but increased the expression of GLUT4, which is mainly responsible for insulin-stimulated glucose uptake. This led us to investigate whether CD2AP plays a role in the regulation of the activity of the insulin signalling pathway or whether it controls glucose transporter trafficking. Interestingly, CD2AP has been reported to be downregulated in mouse podocytes cultured in diabetic conditions and in an experimental model of diabetes *in vivo* (Ha *et al.*, 2015). Also, single-nucleotide polymorphisms (SNPs) in *CD2AP* gene are associated to end-stage renal disease in patients with T1DM (Hyvönen *et al.*, 2013). Thus, we have proposed here that reduced levels of CD2AP and attenuated glucose uptake into podocytes might contribute to podocyte injury.

6.1.1 Insulin signalling through PI3K-AKT pathway is not affected by the absence of CD2AP

CD2AP forms together with nephrin and podocin a complex that activates the PI3K-AKT signalling by binding to the p85 regulatory subunit of PI3K (Huber *et al.*, 2003). Nonetheless, CD2AP does not appear to be crucial for the activation of AKT as insulin stimulated dose-dependent rise of the S473 phosphorylation of AKT in podocytes lacking CD2AP, as we show in Study I and as previously reported (Schiffer *et al.*, 2004). Also, phosphorylation of AS160, the signal to release GLUT4-containing vesicles from the intracellular location to be trafficked to the PM in response to insulin, was not dependent on the presence of CD2AP.

We observed that in the absence of CD2AP, SHIP2, the negative regulator of the PI3K pathway, is upregulated and its activity is increased (Study II), but interestingly, this does not affect insulin-stimulated S473 phosphorylation of AKT in CD2AP-/- podocytes (Study I). This is contradictory to the previous findings showing that overexpression of SHIP2 negatively regulates insulin- and IGF-I- induced signalling through AKT (Hyvönen *et al.*, 2010; Soeda *et al.*, 2010). The explanation behind this contradiction might be in the differences in the overexpression level of SHIP2: In CD2AP-/- podocytes SHIP2 level is upregulated by only 30%, whereas mice and cells

overexpressing SHIP2 reach several-folds higher expression level of SHIP2. Even though the insulin signalling appears functional in the absence of CD2AP, insulin-stimulated GLUT4 translocation to the PM and glucose uptake are disrupted (Study I). This led us to investigate the role of CD2AP in different steps of the GLUT4 trafficking.

6.1.2 Endocytosis of GLUT4 is not affected by the absence of CD2AP

We did not detect any changes in the endocytosis of GLUT4 or transferrin in podocytes lacking CD2AP (Study I), even though both CD2AP and SHIP2 have been reported to play a role in endocytosis. CD2AP has been reported to regulate endocytosis of epidermal growth factor receptors (EGFR) in MDA-MB-231 and HeLa cells (Lynch *et al.*, 2003). Interestingly, SHIP2 knockdown has been shown to lead to EGFR degradation in MDA-231 and HeLa cells (Prasad and Decker, 2005), and to reduce ligand-induced endocytosis of Ephrin A2 receptor in MD231 cells (Prasad, 2009). The endocytic defects have been reported only in cancer cell lines, and podocytes have distinct characteristics compared to these cell lines. In the light of our data, CD2AP does not regulate the endocytosis of GLUT4 in podocytes.

6.1.3 CD2AP is found in GSVs

In CHO cells CD2AP has been shown to control endosome morphology and trafficking (Cormont *et al.*, 2003), and CD2AP knockout mice have been reported to have defects in the formation of multivesicular bodies (Kim *et al.*, 2003). CD2AP also co-localizes in the perinuclear region with COPI, clathrin and Rab5 – all participating in vesicle formation (Havrylov *et al.*, 2008). We found that CD2AP co-fractionates with GSV markers IRAP, sortilin and GLUT4, and that HA-GLUT4-GFP is stuck to the perinuclear area in CD2AP-/- podocytes (Study I). Sortilin controls the formation of GSVs by interacting with IRAP, GLUT4 (Shi and Kandror, 2007) and clathrin adaptor GGA2 (Bilodeau *et al.*, 2004). We observed that in the absence of CD2AP, the expression levels of GSV proteins sortilin and GLUT4 are elevated (Study I). Overexpression of sortilin stabilizes GLUT4 and increases GSV formation, and thus induces glucose uptake in adipocytes (Shi and Kandror 2005). However, in podocytes lacking CD2AP, glucose uptake is disrupted, suggesting that the elevated levels of these proteins are part of a compensatory mechanism, and that the role of CD2AP lies in the re-formation or trafficking of insulin responsive GSVs.

6.1.4 CD2AP plays a role in the formation and sorting of GSVs

We observed that the GSV protein GGA2 (also known as Vear) interacts with CD2AP (Study I). Interestingly, in glomerulus GGA2 is expressed solely in

podocytes (Poussu *et al.*, 2001). GGA2 is not just a clathrin adaptor, as it is also involved in sorting of newly synthetized GLUT4 and GLUT4 internalized from the PM to insulin responsive GSVs (Li and Kandror, 2005). Together the findings reveal that insulin stimulation enhances the interaction between GGA2 and CD2AP, that budding of GSVs from the TGN is not dependent on the presence of CD2AP, and the data obtained from live-cell imaging showing that internalized GLUT4 forms insulin-unresponsive concentrates to the perinuclear region of CD2AP-/- podocytes, suggest a role for CD2AP in sorting of the internalized GLUT4 back to insulin responsive GSVs. This hypothesis is supported by the findings that in the absence of CD2AP, insulin does not stimulate the recycling of clathrin back to donor membranes and it enhances the interaction between GGA2 and clathrin (Study I).

6.1.5 CD2AP is one of the adaptors linking clathrin to actin

We discovered that CD2AP interacts with clathrin (Study I). CD2AP is known to also bind actin directly (Lehtonen *et al.*, 2002) and both CD2AP and SHIP2 have been reported to regulate actin cytoskeleton reorganization (Yaddanapudi *et al.*, 2011; Prasad and Decker 2005). Thus, we hypothesised that CD2AP could link clathrin to actin cytoskeleton and indeed, we observed that actin-clathrin interaction is diminished in the absence of CD2AP. Actin has been suggested to assist the budding of clathrin-coated vesicles from the donor membranes (Carreno *et al.*, 2004), but clustering of GLUT4 to the perinuclear area in the absence of CD2AP (Study I) resembles a previously reported uncoating defect (Pechstein *et al.*, 2015).

6.1.6 CD2AP participates in the regenaration of insulin-responsive GSVs

The association of CD2AP with clathrin and GGA2 suggests that CD2AP controls the sorting of GLUT4 and regeneration of insulin-responsive GSVs. The absence of CD2AP might lead to defective uncoating of clathrin-coated vesicles, thus leading to the accumulation of GLUT4 into cytosol. In the light of our data we suggest that this is a new mechanism by which CD2AP regulates GLUT4 trafficking.

6.2 Role of CD2AP and SHIP2 in podocyte apoptosis

The loss of podocytes strongly predicts the progression of DN (Weil *et al.*, 2012) and this is thought to be due to an increase in apoptosis (Susztak *et al.*, 2006). In addition to defected GLUT4 trafficking, podocytes lacking CD2AP

are more susceptible to apoptosis than wild type cells (Schiffer *et al.*, 2004, Study I and II). Interestingly, we found that in the absence of CD2AP or after PA-treatment leading to diminished levels of CD2AP, both the expression level and activity of its interaction partner SHIP2 are elevated. Overexpression of SHIP2 has been shown to increase apoptosis of podocytes (Hyvönen *et al.*, 2010), neuronal cells (Soeda *et al.*, 2010) and hepatocytes (Gorgani-Firuzjaee *et al.*, 2015). Together these results suggested that the functions of CD2AP and SHIP2 may be interrelated in the regulation of apoptosis of podocytes. Consequently, we set out to investigate the mechanisms responsible for the increased apoptosis caused by the lack of CD2AP and whether inhibition of SHIP2 activity could rescue CD2AP-deficient podocytes from apoptosis.

6.2.1 Absence of CD2AP reduces T308 phosphorylation of AKT

In addition to regulating glucose transport, PI3K-AKT and MAPK signalling pathways are pivotal for anti-apoptotic signalling in podocytes (Welsh *et al.*, 2010). As discussed in part 6.1.1, the overexpression of SHIP2 inhibits the PI3K pathway, but insulin-stimulated S473 phosphorylation of AKT was not disturbed in CD2AP-/- podocytes (Study I), even though they present an elevated level and activity of SHIP2 (Study II). Interestingly, we detected reduced T308 phosphorylation of AKT in the podocytes lacking CD2AP. PDK1 is the enzyme responsible for the phosphorylation of T308 residue in AKT (Alessi *et al.*, 1997) and its knockdown increases podocyte apoptosis by inhibiting anti-apoptotic and boosting pro-apoptotic signalling (Saurus *et al.*, 2015). The decreased AKT phosphorylation on T308 suggested a defected signalling through PDK1 and indeed, the expression of PDK1 was decreased in the absence of CD2AP (Study II). The knockdown of PDK1 has also been shown to reduce the amount of CDK2 and vice versa (Saurus *et al.*, 2016). CDK2 has been shown to activate AKT during the cell cycle progression (Liu *et al.*, 2014). The decreased expression of PDK1 and CDK2 could be observed after the massive decrease of CD2AP expression induced by the treatment with podocyte toxin PA (Study II; Saurus *et al.*, 2016). Prevention of the PA-induced downregulation of PDK1 and CDK2 by overexpression of CD2AP suggests a role for PDK1 and CDK2 in CD2AP-mediated cell survival pathway. The absence of CD2AP has also been shown to downregulate base-line activation of ERK (Tossidou *et al.*, 2007), and overexpression of SHIP2 has been shown to reduce insulin-stimulated activation of ERK (Ishihara *et al.*, 1999). In line with Tossidou *et al.* (2007), we detected reduced phosphorylation of ERK1/2 in the podocytes lacking CD2AP (Study II).

6.2.2 Lack of CD2AP increases the production of ROS

Excess production of ROS results in apoptosis *via* p38 MAPK and caspase-3 (Susztak *et al.*, 2006), and the progression of kidney injury (Inagi, 2010;

Kawakami *et al.*, 2015). We observed that the production of ROS is elevated in cultured podocytes lacking CD2AP and in glomeruli of 3-weeks-old CD2AP-/- mice (Study II). Interestingly, our finding coincides with increased podocyte apoptosis and development of albuminuria in the CD2AP-/- mice (Schiffer *et al.*, 2004). These mice have been reported to show elevated urinary levels of plasminogen, which induces production of ROS in podocytes (Raij *et al.*, 2016) further supporting our findings.

6.2.3 CD2AP protects podocytes from PA-induced increment of ROS and apoptosis

We show that treating cultured human podocytes with PA increases ROS production, downregulates the expression levels of CD2AP, PDK1 and CDK2 and increases apoptosis (Study II). PA-treatment has been previously shown to increase ROS levels (Marshall et al, 2006), apoptosis (Sanwal *et al.*, 2001) and TGF-β (Jones *et al.*, 1992). Overexpression of CD2AP attenuated the PA-induced increase of ROS, prevented the downregulation of PDK1 and CDK2, and rescued podocytes from apoptosis (Study II). This suggests that CD2AP has a role in protecting podocytes from PA-induced increase of ROS and apoptosis. In the absence of CD2AP, the expression of TGF- β and apoptosis have been shown to be elevated *in vivo* (Schiffer *et al.*, 2004). CD2AP is essential for the TGF-β-induced activation of the PI3K-AKT pathway and in the absence of CD2AP, TGF-β activates the pro-apoptotic p38 MAPK pathway instead (Schiffer *et al.*, 2004). Studies on cultured mouse podocytes *in vitro* indicate that the TGF-β-induced apoptosis is coupled with an increase in ROS production (Abe *et al.*, 2013; Das *et al.*, 2014). This suggests that CD2AP is an important mediator of PA-induced podocyte injury, as restoring CD2AP expression in PA-treated podocytes reduced oxidative stress and rescued cells from apoptosis.

6.2.4 Inhibition of SHIP2 decreases the production of ROS but aggravates apoptosis in CD2AP-/- podocytes

We found that inhibition of the catalytic activity of SHIP2 using AS1949490 significantly reduced the production of ROS, but despite this increased apoptosis in podocytes lacking CD2AP (Study II). The decrease of ROS is in line with the report that inhibition of SHIP2 reduces the production of ROS in hepatocytes (Gorgani-Firuzjaee *et al.*, 2015). Inhibition of SHIP2 did not reduce the level of ROS nor had an effect on podocyte apoptosis induced by PA (Study II), apparently because PA is such a potent podocyte toxin and the treatment causes extensive damage in the cells. Also, AS1949490 inhibits only about 35% of the phosphatase activity of SHIP2 in podocytes (Study III), and this might not be enough to negate the effect of PA on an increase in ROS. In addition to SHIP2, there might be other contributors to PA-induced ROS

production (Wang *et al.*, 2009) or SHIP2 might not even play a role in the process.

To find an explanation why inhibition of SHIP2 increased apoptosis in the absence of CD2AP, we investigated whether SHIP2 inhibition affects AKT and ERK signalling pathways. AS1949490 treatment increased the phosphorylation of PDK1 regardless of the expression status of CD2AP. However, we observed that AS1949490 increased phosphorylation of only T308 but not S473 of AKT in the absence of CD2AP (Study II). This indicates that CD2AP-SHIP2 interaction is important for the regulation of S473 phosphorylation of AKT, and thus, for full activation of AKT (Alessi *et al.*, 1996). The expression status of CD2AP did not affect the increment in ERK phosphorylation in response to SHIP2 inhibitor treatment. In lymphatic endothelial cells AS1949490 has been reported to increase the phosphorylation of AKT and ERK, and knockdown of SHIP2 has been shown to increase the apoptosis of these cells (Agollah *et al.*, 2014). Even though ERK activation is considered to be anti-apoptotic, it can also be a pro-apoptotic signal (reviewed by Cagnol and Chambard, 2009; and in Lu and Xu, 2006).

6.2.5 Role of CD2AP and SHIP2 in podocyte apoptosis

Our data confirms previous observations showing that CD2AP protects podocytes from apoptosis (Raij, *et al.*, 2016; Schiffer *et al.*, 2004) and shows that overexpression of CD2AP protects cultured human podocytes against PA-induced apoptosis. Reflecting our current results and previous findings revealing that CD2AP binds to the inactive, nonphosphorylated form of SHIP2 (Hyvönen *et al.*, 2010), we suggest that CD2AP has an inhibitory role on SHIP2 activity. Our data further implies that the CD2AP-SHIP2 interaction, or at least the presence of CD2AP, is important for the full activation of AKT in response to AS1949490 treatment. Determining how the balance of AKT and ERK phosphorylation activate anti-apoptotic or pro-apoptotic pathways needs further study.

6.3 Inhibition of the catalytic activity of SHIP2 restores insulin sensitivity and protects podocytes from apoptosis

Inhibition of SHIP2 with small-molecule inhibitors AS1949490 and AS1938909 has been reported to increase insulin sensitivity both *in vitro* and *in vivo* (Suwa *et al.*, 2009 and 2010b). Notably, insulin sensitizers have been suggested to have a renoprotective effect on diabetic patients (Miyazaki *et al.*, 2007). Here we show that metformin inhibits SHIP2 and protects podocytes from apoptosis in culture (Study III). We further show that treatment of T2DM

patients with metformin associates with reduced podocyte loss. SHIP2 inhibition has been shown to protect hepatocytes from apoptosis (Gorgani-Firuzjaee *et al.*, 2015) and to boost proliferation in gastric cancer cells (Ye *et al.*, 2016). Yet, in other cancers inhibition of SHIP2 has been reported to promote apoptosis as in MDB-21 breast cancer cells (Fuhler *et al.*, 2012) and prostate cancer cell lines lacking PTEN (Lincova *et al.*, 2009). Thus, the effects of SHIP2 inhibition on apoptosis and cell proliferation appear to be both cell type- and context-dependent.

6.3.1 Metformin inhibits SHIP2

Previous studies have reported screening of SHIP2 inhibitors by using high-throughput and ligand-based virtual screening methods, but the molecules identified had poor drug-like properties, including poor solubility and bioavailability and generally poor pharmacokinetical properties (Suwa *et al.*, 2010a). The X-ray chrystallocraphy structure of the phosphatase domain of SHIP2 was first uploaded to Protein Data Bank in 2010 (Tresaugues *et al.*, 2014) and the active site was revealed in 2012 (Mills *et al.*, 2012). These findings allowed us to use structure-based virtual screening method to find novel SHIP2 inhibitors. Surprisingly, we found that an old anti-diabetic drug metformin was one of the top candidates to bind SHIP2 *in silico*. We further found that metformin was equally potent and specific SHIP2 inhibitor as AS1949490 *in vitro* (Study III). This supports the hypothesis presented in the literature that SHIP2 is a good target for the treatment of T2DM (Sasaoka *et al.*, 2006). In line with the finding that the inhibition of the catalytical activity of SHIP2 induces apoptosis in cancer cells, as discussed above, we found a few known drugs used in the treatment of cancer as potent SHIP2 inhibitors (Study III). Interestingly, metformin has been reported to lower the risk of cancer in T2DM patients (Kusturica *et al.*, 2017) supporting the potential of metformin, and potentially other SHIP2 inhibitors, as a treatment for both cancer and T2DM.

6.3.2 Metformin, but not AMPK activator AICAR, increases glucose uptake in podocytes

We demonstrated that the effect of metformin to enhance glucose uptake occurs *via* SHIP2 inhibition, as the knockdown and overexpression of SHIP2 attenuated the capability of metformin to enhance glucose uptake in cultured myotubes (Study III). Parallel to SHIP2 inhibitors AS1949490 and AS1938909, metformin has been previously reported to enhance glucose uptake (Rogacka *et al.*, 2014; Turban *et al.*, 2012; Suwa *et al.*, 2009, 2010b). As metformin has also been shown to act as an AMPK activator in muscle cells, although the data is controversial (Ohno *et al.*, 2015; Rogacka *et al.*, 2014; Turban *et al.*, 2012; Foretz *et al.*, 2010), we studied the activation status of

AMPK and the capability of a specific AMPK activator, AICAR, to induce glucose uptake in podocytes. Even though we detected that metformin activates AMPK *in vitro*, the specific activation of AMPK using AICAR did not increase glucose uptake of podocytes (Study III). A short-term treatment of db/db mice with AS1949490 has been shown to reduce the fasting blood glucose level of the mice and improve their insulin sensitivity (Suwa *et al.*, 2009). Similar short-term treatment with metformin was reported not to reduce the fasting blood glucose level of db/db mice (Eskens *et al.*, 2013, Study III), but we observed improved insulin sensitivity of the db/db mice in our study (Study III). Curiously, we did not observe activation of AMPK in the tissues of the db/db mice that were treated with metformin (Study III). Together these results suggest that metformin improves insulin sensitivity and glucose uptake in peripheral tissues by acting as a SHIP2 inhibitor, and not *via* activation of AMPK in cultured podocytes or db/db mice.

6.3.3 Metformin enhances glucose uptake by slowing down endocytosis of GLUT4

In search for the mechanism by which metformin enhances glucose uptake, we first analyzed phosphorylation of AKT as SHIP2 acts as a negative regulator of the PI3K/AKT pathway by hydrolysing $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (Ishihara *et al.*, 1999; Pesesse *et al.*, 1997), and as overexpression of SHIP2 reduces the phosphorylation of AKT (Hyvönen *et al.*, 2010; Wada *et al.*, 2001). Metformin did not induce the phosphorylation of AKT alone or together with insulin (Study III), indicating that the mechanism of metformin-stimulated glucose uptake is not mediated *via* activation of the PI3K-AKT pathway. This is in line with previously published data on metformin (Kristensen *et al.*, 2014). However, inhibition of the catalytic activity of SHIP2 with small-molecule inhibitors AS1949490 and AS1938909 has been shown to increase the phosphorylation of AKT after insulin stimulation (Ichihara *et al.*, 2013; Suwa *et al.*, 2009) and in basal state (Study II). The explanation behind this contradiction remains to be elucidated.

SHIP2 has been reported have a role in the regulation of actin cytoskeleton organization, distribution of endocytic vesicles (Prasad and Decker, 2005) and formation of clathrin coated pits/vesicles (Nakatsu *et al.*, 2010). All these processes are linked to glucose transporter trafficking. Furthermore, metformin is known to regulate GLUT trafficking *via* affecting the configuration and/or interaction of proteins at the PM (Wiernsperger, 1999). We found that metformin increases GLUT4 levels at the PM and slows down GLUT4 endocytosis (Study III). Also, AS1949490 treatment of myocytes slowed down the endocytosis of GLUT4 (Tolvanen *et al.*, Unpublished). This might be due to inbalanced phosphoinositides at the PM, as increased level of $PI(4,5)P_2$, a substrate of SHIP2 (Nakatsu et al., 2010), has been shown to

reduce the endocytosis of GLUT4 (Kanzaki *et al.*, 2004). Together with our data this supports our hypothesis that metformin enhances peripheral glucose uptake *via* the inhibition of SHIP2.

6.3.4 Metformin does not inhibit SHIP2 in cultured hepatocytes or in liver in db/db

We observed that metformin inhibits the activity of SHIP2 in skeletal muscle and kidney both *in vitro* and *in vivo*, but not in cultured hepatocytes or in liver of db/db mice (Study III). Curiously, similarly as metformin, SHIP2 inhibitors AS1949490 and CPDA reduce the expression of PCK1, one of the rate-limiting enzymes of gluconeogenesis, in db/db mice (Ichihara *et al.*, 2013; Study III). However, the activity of SHIP2 was not measured by Ichihara *et al.*, leaving open whether the inhibitors acted directly *via* inhibition of SHIP2. In fact, we observed in our own studies that AS1949490 does not inhibit SHIP2 activity in cultured hepatocytes (Tolvanen *et al.*, Unpublished). This proposes alternative explanations for the ineffectiveness of metformin to inhibit SHIP2 activity in liver and hepatocytes: (1) Metformin and AS1949490 may suppress gluconeogenesis *via* another target/targets. In line with this, metformin has been reported to directly bind to and inhibit mitochondrial glycerophosphate dehydrogenase, which suppresses gluconeogenesis in liver (Madiraju *et al.*, 2014). (2) The conformation of SHIP2 could differ in liver from the conformation in muscle and kidney and thus the inhibitor might be washed away from SHIP2 during our activity measurements using liver tissue. The rationale behind the latter explanation is theoretical: The conformation of the protein can change due to its binding to other proteins or different ligands. The most likely structural feature to change the conformation of the protein is a random-coil, and the active sites of the proteins are usually located to areas containing random-coil structures (Alberts *et al.*, 2002; Höltje *et al.*, 2003). The conformation of SHIP2 could be different in different cell types/tissues or could change differently after the binding of the inhibitors in liver compared to skeletal muscle or podocytes, and this could weaken the non-covalent binding energies keeping the inhibitor bound to the active site. If the binding energies are insufficient to hold the inhibitor bound to the active site throughout the activity measurement, the inhibitor could be washed away and thus would fail in inhibiting the activity of the target protein.

6.3.5 Metformin associcates with reduced podocyte loss in glomeruli of T2DM patients *via* inhibition of SHIP2

As noted in the section 6.3, insulin sensitising drugs show renoprotective effects in patients with diabetes and SHIP2 inhibition protects against apoptosis in hepatocytes (Gorgani-Firuzjaee *et al.*, 2015; Miyazaki *et al.*, 2007). The effects of SHIP2 inhibition on PI3K-AKT and MAPK signalling are

discussed in detail in sections 6.2.1 and 6.2.4. Metformin treatment rescued podocytes from SHIP2 overexpression-induced apoptosis by restoring the activity of the PI3K-AKT pathway (Study III). This finding is in line with the reports showing that metformin prevents podocyte injury in a rat model of T2DM (Kim *et al.*, 2012), and reduces podocyte apoptosis induced by high glucose *in vitro* (Langer *et al.*, 2016). The overexpression of SHIP2 is a feasible model to investigate its effects in diabetic conditions as the level of SHIP2 has been shown to be upregulated in various tissues of rodent models of T2DM (Hyvönen *et al.*, 2010; Hori *et al.*, 2002). Interestingly, we did not detect an increased expression level of SHIP2 in the kidneys of patients with T2DM compared to people without diabetes (Study III). However, we observed that the activity of SHIP2 was higher in the kidneys of patients with T2DM receiving non-metformin medication compared to people without diabetes. Notably, the activity of SHIP2 in the kidneys did not differ between patients with T2DM receiving metformin medication and people without diabetes (Study III). We also observed that the patients with T2DM receiving metformin presented significantly higher podocyte counts per glomerulus than the patients with T2DM receiving non-metformin medication (Study III). Previous studies show that podocyte apoptosis is associated with progressive podocyte depletion in patients with diabetes, and when podocyte loss exceeds 40%, the progression of DN to end-stage renal disease is guaranteed (Verzola *et al.*, 2007; Wharram *et al.*, 2005). These data suggest that metformin protects patients with T2DM from podocyte loss *via* the inhibition of SHIP2 and thus may prevent the progression of DN at an early stage.

6.3.6 Ability of metformin to inhibit SHIP2 validates SHIP2 as a potential drug target for treating T2DM

We identified SHIP2 as a novel target of metformin and confirmed that metformin inhibits the phosphatase activity of SHIP2 *in vitro* and *in vivo* in muscle and kidney, thus improving insulin sensitivity. We also detected that in the kidneys of patients with T2DM receiving metformin, the activity of SHIP2 was not statistically different from people without diabetes, in contrast to the patients with T2DM receiving non-metformin medication. This might be one of the reasons for the decreased podocyte loss in the kidneys of T2DM patients receiving metformin. Thus, we suggest that SHIP2 is a feasible drug target to treat insulin resistance in patients with T2DM and DN.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis provides new insights into the role of CD2AP as a regulator of glucose transporter trafficking, documents the interrelationship of CD2AP and SHIP2 in podocyte apoptosis, and describes a novel mechanism of action for metformin.

Since insulin resistance and podocyte apoptosis are linked to the development and progression of diabetic nephropathy, understanding the molecular mechanisms underlying both processes is paramount to finding novel treatments for DN. We found that the lack of CD2AP disrupts the insulin-induced glucose uptake in podocytes and this is due to reduced trafficking of GSVs from the perinuclear region to the plasma membrane. We also showed that CD2AP cofractionates with components the of GSVs and associates with clathrin and GGA2. Together our data suggest a new mechanism by which CD2AP controls sorting of GLUT4 and regeneration of insulin-responsive GSVs. Further studies are still needed to clarify whether there is a defect in clathrin coating of GSVs as we discussed in part 6.1.5. As SHIP2 is known to play a role in the formation and uncoating of clathrin-coated vesicles, and as we show an interplay between CD2AP and SHIP2, this raises questions of the interconnected role of these proteins in GSV coating and trafficking. This could be studied utilizing SHIP2 inhibitors in the absence and presence of CD2AP and monitoring the effects on GSV formation and trafficking.

Further to the lack of CD2AP causing defects in glucose transport, it also makes the podocytes more prone to undergo apoptosis. We discovered that in the absence of CD2AP the oxidative stress is increased in podocytes, which could be contributing to the increased apoptosis. We further found that inhibition of SHIP2 with AS1949490 reduces ROS production caused by the absence of CD2AP, but this was not enough to rescue the cells from apoptosis. It would be interesting to study wether metformin produces a similar phenotype in CD2AP-/- podocytes as AS1949490. We also detected downregulation of CD2AP expression and an increase in the production of ROS after treating podocytes with PA, a podocyte toxin. To give a definitive answer whether the increased ROS level itself decreases the expression of CD2AP and whether overexpression of CD2AP prevents purely ROS-induced apoptosis, we would need to conduct further studies treating podocytes with oxidisers producing ROS, such as hydrogen peroxide. The search for the mechanism underlying SHIP2 inhibition-induced increment of podocyte

apoptosis in the absence of CD2AP led to the finding that in CD2AP-/- podocytes, the inhibition of SHIP2 is not able to fully activate AKT or ERK in comparison to WT podocytes. These impairments in anti-apoptotic signalling pathways could in fact activate pro-apoptotic signalling pathways, as activation of ERK has been associated with increased apoptosis. Further studies are required to elucidate the role of partial activation of AKT together with mild activation of ERK in podocyte apoptosis. It would be interesting to investigate this with modified versions of AKT with different phosphorylation statuses of T308 and S473. Our finding, showing that inhibition of SHIP2 in the absence of CD2AP can lead to increased podocyte apoptosis, is important in the respect of patient safety at the point of novel SHIP2 inhibitors being launched to the market, as some kidney diseases are associated with reduced expression or lack of CD2AP. This, together with our discovery that metformin inhibits SHIP2, could also explain why metformin causes severe kidney failure in few patients who already suffer from reduced kidney function. To be more confident about this, we should first show that metformin treatment leads to a similar phenotype as AS1949490 treatment in podocytes lacking CD2AP. Also, further studies investigating the levels of CD2AP, even though challenging in humans, would be needed to support this hypothesis. In Study III, we show that in general metformin is a good drug for the treatment of T2DM at the perspective of kidney. Data obtained from the patient material clearly indicates that metformin, and thus potentially also other SHIP2 inhibitors, may be beneficial for preventing the progression of DN in T2DM patients.

Our studies, as well as previous studies, have shown that SHIP2 is a good target for the treatment of insulin resistance and T2DM, but the known SHIP2 inhibitors have poor solubility and pharmacokinetic properties. We sought for novel SHIP2 inhibitors using structure-based virtual screening, and found several novel inhibitors. In Study III we show that metformin inhibits the activity of SHIP2 both *in vitro* and *in vivo*, and thus, we suggest that the mechanism by which metformin abrogates peripheral insulin resistance occurs by the inhibition of SHIP2 activity. As metformin is already used for the treatment of insulin resistance and T2DM, the finding that it acts as a SHIP2 inhibitor further validates the potential of SHIP2 as an excellent target for treating insulin resistance and T2DM. The most important outcomes of our virtual screening were the unknown molecules that we found to inhibit SHIP2, some of which are much more potent SHIP2 inhibitors than metformin. Further studies are needed to determine the pharmacological properties of these molecules as well as their toxicities. The structures of these molecules can be optimized aiming for maximal inhibition of the SHIP2 activity together with close to sub-micromolar IC_{50} values and better pharmacokinetic properties. Once having achieved these goals, we will be able to apply for a

patent to protect our findings. With the best prospects, we can proceed to pre-clinal testing and potentially in the future provide a new medicine for the treatment of T2DM and DN.

The studies conducted in cells cultured on a petridish have their limitations as in the organs *in vivo*, these cells interact with other cell types and do not grow on flat surfaces. Culturing the cells in 3D environment using different matrixes works for some cell types, but still the interaction with other cell types is missing. There has been effort to develop methods to create artificial organoids like the glomerulus by culturing fenestrated endothelial cells on magnetic beads and adding podocytes to grow on top of them. In these small sferes researchers have seen the formation of GBM between the cell types as seen in real glomerulus. In future, genetically modifying or chemically challenging these cell types individually or together could reflect in a better fashion the complex synergic biological prosecesses. These organoid models could be more easily controlled and studied at the molecular level than the costly animal models.

The main reason behind the ever-expanding epidemic of T2DM is the western life style with an endless supply of cheap calorie-dense food and too little of physical exercise. During evolution our bodies have adapted to shortage of food and high physical activity, thus making it possible for humans to survive in harsh environments and to populate the whole globe. Just considering these facts, it sounds easy to get rid of T2DM: just eat less and exercise more. Unfortunately, these adaptations have made us grave for and get addicted to calorie-dense food, and to efficiently store all the excess energy as fat. As the SHIP2 knock-out mice are resistant to high-fat diet induced obesity, also SHIP2 inhibitors could have anorexic properties. If this would be the case, these inhibitors could be used for preventing and potentially treating obesity. Already better control of the blood glucose level of patients with T2DM would prevent macro-vascular and other micro-vascular complications in addition to DN.

8 ACKNOWLEDGEMENTS

This study was carried out at the Department of Pathology, University of Helsinki, during 2010-2017. I would like to thank the head of the department Professor Tom Böhling, and his predecessors, for providing me with excellent research facilities and pleasant, stimulating atmosphere to work.

I would like to thank the reviewers of the thesis, Professor Jukka Hakkola and Professor Seppo Vainio, for their scarification of time and effort to go through my work, and for the constructive comments to improve the thesis.

I want to express my gratitude to my supervisor Assistant Professor Sanna Lehtonen. She has been guiding and supporting me through these projects and taught a lot to me. I want to thank her for letting me to take part in planning and designing the projects. Her door was always open, and she had always time to answer my questions and have interesting conversations about science.

The members of the follow-up group of my thesis, Professor Ville Hietakangas and Professor Hannu Sariola, are gratefully acknowledged for their valuable comments and support.

I wish to express my gratitude to all collaborators and co-authors in these studies. From the Department of Chemistry Professor Kristiina Wähälä and Mika Berg the chemical synthesis wizards. The expert pathologist and clinicians Per-Henrik Groop, Eero Lehtonen, Tuomas Mirtti, Harry Nisen and Jukka Tienari are acknowledged for all their help and for providing the human material. From our group I would like to thank Surjya Dash, Vincent Dumont, Sara Kuusela, Mervi Latvala, Sanna Lehtonen, Sonja Lindfors, Zydrune Polianskyte-Prause, Pauliina Koreneff os. Saurus and Hong Wang. I want to specially thank Zydrune for sharing with me the good and bad times with the SHIP2 inhibitor project.

I am extremely grateful to Professor Toshiyasu Sasaoka providing me the chance to visit his laboratory in the University of Toyama, Japan. I would like to thank also all the members of the group, who were very helpful and friendly. Special acknowledgements to Associate Professor Hiroshi Tsuneki and Assistant Professor Tsutomu Wada, who assisted us with all the practical things and taught us about Japanese culture in our visits to Kanazawa and Shirakawa-go.

High Throughput Biomedicine Unit, Biomedicum Imaging Unit, Biomedicum Flow Cytometry Unit and CSC Finland. I'm grateful for the facilities equipment, computational power and expertise provided by all these services.

I want to acknowledge Niina Ruoho, Leena Saikko and Tiiu Arumäe for all the technical assistance in experiments and keeping the lab stocked with reagents during all these year.

Thanks for all the current and previous members of Sanna Lehtonen lab, Surjya Dash, Vincent Dumont, Laura Hautala, Eija Heikkilä, Mervi Hyvönen, Pauliina Koreneff, Sara Kuusela, Sonja Lindfors, Jette-Brit Naams, Zydrune Polianskyte-Prause, Mervi Ristola, Niina Ruoho, Leena Saikko, Neeta Sengupta, Hong Wang and Anita Wasik. Special thanks to Vincent, Sara, Niina, Mervi H. and Anita, as I'm proud to call you as my friends, not just colleges. Without the perceptiveness of Niina and Sara I most probably would not be here today, so the words are not enough to thank you for that.

All my friends, the academic ones and the most who are not academics, thanks you for your mental support and helping me to think something else than my research. And now you can thank or curse me, as my work did not start zombie apocalypse (yet).

I thank my parents Markku and Riitta and my sister Pihla for all their support to my studies and other aspects of life. I also thank my sister-in-law Johanna and her family for the company on our skiing trips to Lapland. Special thanks for my other sister-in-law Niina, who checked the language and grammar of this book.

Finally, I thank my beloved wife Mari Pitkänen for her understanding, help and mental support during these years.

These studies were financially supported by the Doctoral Programme in Biomedicine, Diabetestutkimussäätiö, European Research Council, Academy of Finland, Jane ja Aatos Erkon säätiö, Sigrid Juselius stiftelse, Päivikki and Sakari Sohlberg Foundation, European Foundation for the Study of Diabetes and Boehringer Ingelheim.

Helsinki, November 2017

Tuomas Tolvanen

9 REFERENCES

Abe, Y., Sakairi, T., Beeson, C. and Kopp, J. B. (2013). TGF-beta1 stimulates mitochondrial oxidative phosphorylation and generation of reactive oxygen species in cultured mouse podocytes, mediated in part by the mTOR pathway. *Am. J. Physiol. Renal Physiol.* **305,** 1477.

Abrahamson, D. R., Hudson, B. G., Stroganova, L., Borza, D. B. and St John, P. L. (2009). Cellular origins of type IV collagen networks in developing glomeruli. *J. Am. Soc. Nephrol.* **20,** 1471-1479.

Adler, A. I., Stevens, R. J., Manley, S. E., Bilous, R. W., Cull, C. A., Holman, R. R. and UKPDS GROUP. (2003). Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int.* **63,** 225-232.

Agollah, G. D., Gonzalez-Garay, M. L., Rasmussen, J. C., Tan, I. C., Aldrich, M. B., Darne, C., Fife, C. E., Guilliod, R., Maus, E. A., King, P. D. et al. (2014). Evidence for SH2 domain-containing 5'-inositol phosphatase-2 (SHIP2) contributing to a lymphatic dysfunction. *PLoS One* **9,** e112548.

Alberts, B., Johnson Alexander, Lewis Julian, Raff Martin, Roberts Keith and Walter Peter. (2002). *Molecular Biology of the Cell*. New York, NY [u.a.]: Garland Science.

Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15,** 6541-6551.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase balpha. *Curr. Biol.* **7,** 261-269.

Amador-Licona, N., Guizar-Mendoza, J., Vargas, E., Sanchez-Camargo, G. and Zamora-Mata, L. (2000). The short-term effect of a switch from glibenclamide to metformin on blood pressure and microalbuminuria in patients with type 2 diabetes mellitus. *Arch. Med. Res.* **31,** 571-575.

Annis, D. A., Cheng, C. C., Chuang, C. C., McCarter, J. D., Nash, H. M., Nazef, N., Rowe, T., Kurzeja, R. J. and Shipps, G. W. (2009).

Inhibitors of the lipid phosphatase SHIP2 discovered by high-throughput affinity selection-mass spectrometry screening of combinatorial libraries. *Comb. Chem. High Throughput Screen.* **12,** 760-771.

Avruch, J. (1998). Insulin signal transduction through protein kinase cascades. *Mol. Cell. Biochem.* **182,** 31-48.

Baelde, H. J., Eikmans, M., Doran, P. P., Lappin, D. W., de Heer, E. and Bruijn, J. A. (2004). Gene expression profiling in glomeruli from human kidneys with diabetic nephropathy. *Am. J. Kidney Dis.* **43,** 636-650.

Baell, J. B. and Holloway, G. A. (2010). New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **53,** 2719-2740.

Baranov, M. V., Revelo, N. H., Dingjan, I., Maraspini, R., Ter Beest, M., Honigmann, A. and van den Bogaart, G. (2016). SWAP70 organizes the actin cytoskeleton and is essential for phagocytosis. *Cell. Rep.* **17,** 1518-1531.

Bastard, J. P., Maachi, M., Lagathu, C., Kim, M. J., Caron, M., Vidal, H., Capeau, J. and Feve, B. (2006). Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur. Cytokine Netw.* **17,** 4-12.

Betz, B. and Conway, B. R. (2014). Recent advances in animal models of diabetic nephropathy. *Nephron Exp. Nephrol.* **126,** 191-195.

Bilodeau, P. S., Winistorfer, S. C., Allaman, M. M., Surendhran, K., Kearney, W. R., Robertson, A. D. and Piper, R. C. (2004). The GAT domains of clathrin-associated GGA proteins have two ubiquitin binding motifs. *J. Biol. Chem.* **279,** 54808-54816.

Bjorn, S. F., Bangstad, H. J., Hanssen, K. F., Nyberg, G., Walker, J. D., Viberti, G. C. and Osterby, R. (1995). Glomerular epithelial foot processes and filtration slits in IDDM patients. *Diabetologia* **38,** 1197-1204.

Blot, V. and McGraw, T. E. (2006). GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. *EMBO J.* **25,** 5648-5658.

Bogan, J. S., Rubin, B. R., Yu, C., Loffler, M. G., Orme, C. M., Belman, J. P., McNally, L. J., Hao, M. and Cresswell, J. A. (2012). Endoproteolytic cleavage of TUG protein regulates GLUT4 glucose transporter translocation. *J. Biol. Chem.* **287,** 23932-23947.

Brett, T. J., Traub, L. M. and Fremont, D. H. (2002). Accessory protein recruitment motifs in clathrin-mediated endocytosis. *Structure* **10,** 797-809.

Caramori, M. L., Fioretto, P. and Mauer, M. (2000). The need for early predictors of diabetic nephropathy risk: Is albumin excretion rate sufficient? *Diabetes* **49,** 1399-1408.

Carreno, S., Engqvist-Goldstein, A. E., Zhang, C. X., McDonald, K. L. and Drubin, D. G. (2004). Actin dynamics coupled to clathrin-coated vesicle formation at the trans-golgi network. *J. Cell Biol.* **165,** 781-788.

Chen, Y., Ma, H., Zhu, D., Zhao, G., Wang, L., Fu, X. and Chen, W. (2017). Discovery of novel insulin sensitizers: Promising approaches and targets. *PPAR Res.* **2017,** 8360919.

Cormont, M., Meton, I., Mari, M., Monzo, P., Keslair, F., Gaskin, C., McGraw, T. E. and Le Marchand-Brustel, Y. (2003). CD2AP/CMS regulates endosome morphology and traffic to the degradative pathway through its interaction with Rab4 and c-cbl. *Traffic* **4,** 97-112.

Coward, R. J., Welsh, G. I., Koziell, A., Hussain, S., Lennon, R., Ni, L., Tavare, J. M., Mathieson, P. W. and Saleem, M. A. (2007). Nephrin is critical for the action of insulin on human glomerular podocytes. *Diabetes* **56,** 1127-1135.

Coward, R. J., Welsh, G. I., Yang, J., Tasman, C., Lennon, R., Koziell, A., Satchell, S., Holman, G. D., Kerjaschki, D., Tavare, J. M. et al. (2005). The human glomerular podocyte is a novel target for insulin action. *Diabetes* **54,** 3095-3102.

Daehn, I., Casalena, G., Zhang, T., Shi, S., Fenninger, F., Barasch, N., Yu, L., D'Agati, V., Schlondorff, D., Kriz, W. et al. (2014). Endothelial mitochondrial oxidative stress determines podocyte depletion in segmental glomerulosclerosis. *J. Clin. Invest.* **124,** 1608-1621.

Das, R., Xu, S., Quan, X., Nguyen, T. T., Kong, I. D., Chung, C. H., Lee, E. Y., Cha, S. K. and Park, K. S. (2014). Upregulation of mitochondrial Nox4 mediates TGF-beta-induced apoptosis in cultured mouse podocytes. *Am. J. Physiol. Renal Physiol.* **306,** 155.

Dash, S. N., Lehtonen, E., Wasik, A. A., Schepis, A., Paavola, J., Panula, P., Nelson, W. J. and Lehtonen, S. (2014). Sept7b is essential for pronephric function and development of left-right asymmetry in zebrafish embryogenesis. *J. Cell. Sci.* **127,** 1476-1486.

Delea, T. E., Edelsberg, J. S., Hagiwara, M., Oster, G. and Phillips, L. S. (2003). Use of thiazolidinediones and risk of heart failure in people with type 2 diabetes: A retrospective cohort study. *Diabetes Care* **26,** 2983-2989.

Dombrowski, F., Klotz, L., Bannasch, P. and Evert, M. (2007). Renal carcinogenesis in models of diabetes in rats: Metabolic changes are closely related to neoplastic development. *Diabetologia* **50,** 2580-2590.

Dubois, E., Jacoby, M., Blockmans, M., Pernot, E., Schiffmann, S. N., Foukas, L. C., Henquin, J. C., Vanhaesebroeck, B., Erneux, C. and Schurmans, S. (2012). Developmental defects and rescue from glucose intolerance of a catalytically-inactive novel Ship2 mutant mouse. *Cell. Signal.* **24,** 1971-1980.

Dustin, M. L., Olszowy, M. W., Holdorf, A. D., Li, J., Bromley, S., Desai, N., Widder, P., Rosenberger, F., van der Merwe, P A, Allen, P. M. et al. (1998). A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* **94,** 667-677.

Ekstrand, A. V., Groop, P. H. and Gronhagen-Riska, C. (1998). Insulin resistance precedes microalbuminuria in patients with insulin-dependent diabetes mellitus. *Nephrol. Dial. Transplant.* **13,** 3079-3083.

El-Jack, A. K., Kandror, K. V. and Pilch, P. F. (1999). The formation of an insulin-responsive vesicular cargo compartment is an early event in 3T3-L1 adipocyte differentiation. *Mol. Biol. Cell* **10,** 1581-1594.

Epstein, P. N., Overbeek, P. A. and Means, A. R. (1989). Calmodulin-induced early-onset diabetes in transgenic mice. *Cell* **58,** 1067-1073.

Eskens, B. J., Zuurbier, C. J., van Haare, J., Vink, H. and van Teeffelen, J. W. (2013). Effects of two weeks of metformin treatment on whole-body glycocalyx barrier properties in db/db mice. *Cardiovasc. Diabetol.* **12,** 175.

Fiorentino, T. V., Prioletta, A., Zuo, P. and Folli, F. (2013). Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Curr. Pharm. Des.* **19,** 5695-5703.

Foretz, M., Guigas, B., Bertrand, L., Pollak, M. and Viollet, B. (2014). Metformin: From mechanisms of action to therapies. *Cell. Metab.* **20,** 953-966.

Foretz, M., Hebrard, S., Leclerc, J., Zarrinpashneh, E., Soty, M., Mithieux, G., Sakamoto, K., Andreelli, F. and Viollet, B. (2010). Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest.* **120,** 2355-2369.

Forsblom, C. M., Groop, P. H., Ekstrand, A. and Groop, L. C. (1992). Predictive value of microalbuminuria in patients with insulin-dependent diabetes of long duration. *BMJ* **305,** 1051-1053.

Fuhler, G. M., Brooks, R., Toms, B., Iyer, S., Gengo, E. A., Park, M. Y., Gumbleton, M., Viernes, D. R., Chisholm, J. D. and Kerr, W. G. (2012). Therapeutic potential of SH2 domain-containing inositol-5'-phosphatase 1 (SHIP1) and SHIP2 inhibition in cancer. *Mol. Med.* **18,** 65-75.

Fujita, H., Hatakeyama, H., Watanabe, T. M., Sato, M., Higuchi, H. and Kanzaki, M. (2010). Identification of three distinct functional sites of insulin-mediated GLUT4 trafficking in adipocytes using quantitative single molecule imaging. *Mol. Biol. Cell* **21,** 2721-2731.

Funaki, M., Randhawa, P. and Janmey, P. A. (2004). Separation of insulin signaling into distinct GLUT4 translocation and activation steps. *Mol. Cell. Biol.* **24,** 7567-7577.

Gatica, R., Bertinat, R., Silva, P., Kairath, P., Slebe, F., Pardo, F., Ramirez, M. J., Slebe, J. C., Campistol, J. M., Nualart, F. et al. (2015). Over-expression of muscle glycogen synthase in human diabetic nephropathy. *Histochem. Cell Biol.* **143,** 313-324.

Gorgani-Firuzjaee, S., Adeli, K. and Meshkani, R. (2015). Inhibition of SH2-domain-containing inositol 5-phosphatase (SHIP2) ameliorates palmitate induced-apoptosis through regulating akt/FOXO1 pathway and ROS production in HepG2 cells. *Biochem. Biophys. Res. Commun.* **464,** 441-446.

Gorgani-Firuzjaee, S. and Meshkani, R. (2015). SH2 domain-containing inositol 5-phosphatase (SHIP2) inhibition ameliorates high glucose-induced de-novo lipogenesis and VLDL production through regulating AMPK/mTOR/SREBP1 pathway and ROS production in HepG2 cells. *Free Radic. Biol. Med.* **89,** 679-689.

Gosmanov, A. R., Wall, B. M. and Gosmanova, E. O. (2014). Diagnosis and treatment of diabetic kidney disease. *Am. J. Med. Sci.* **347,** 406-413.

Govers, R., Coster, A. C. and James, D. E. (2004). Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. *Mol. Cell. Biol.* **24,** 6456-6466.

Gropp, M., Itsykson, P., Singer, O., Ben-Hur, T., Reinhartz, E., Galun, E. and Reubinoff, B. E. (2003). Stable genetic modification of human embryonic stem cells by lentiviral vectors. *Mol. Ther.* **7,** 281-287.

Gross, J. L., de Azevedo, M. J., Silveiro, S. P., Canani, L. H., Caramori, M. L. and Zelmanovitz, T. (2005). Diabetic nephropathy: Diagnosis, prevention, and treatment. *Diabetes Care* **28,** 164-176.

Guo, J. K., Marlier, A., Shi, H., Shan, A., Ardito, T. A., Du, Z. P., Kashgarian, M., Krause, D. S., Biemesderfer, D. and Cantley, L. G. (2012). Increased tubular proliferation as an adaptive response to glomerular albuminuria. *J. Am. Soc. Nephrol.* **23,** 429-437.

Guzman, J., Jauregui, A. N., Merscher-Gomez, S., Maiguel, D., Muresan, C., Mitrofanova, A., Diez-Sampedro, A., Szust, J., Yoo, T. H., Villarreal, R. et al. (2014). Podocyte-specific GLUT4-deficient mice have fewer and larger podocytes and are protected from diabetic nephropathy. *Diabetes* **63,** 701-714.

Ha, T. S., Hong, E. J. and Han, G. D. (2015). Diabetic conditions downregulate the expression of CD2AP in podocytes via PI3-K/akt signalling. *Diabetes Metab. Res. Rev.* **31,** 50-60.

Hale, L. J., Welsh, G. I., Perks, C. M., Hurcombe, J. A., Moore, S., Hers, I., Saleem, M. A., Mathieson, P. W., Murphy, A. J., Jeansson, M. et al. (2013). Insulin-like growth factor-II is produced by, signals to and is an important survival factor for the mature podocyte in man and mouse. *J. Pathol.* **230,** 95-106.

Hampp, C. and Pippins, J. (2017). Pioglitazone and bladder cancer: FDA's assessment. *Pharmacoepidemiol. Drug Saf.* **26,** 117-118.

Haraldsson, B., Nystrom, J. and Deen, W. M. (2008). Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol. Rev.* **88,** 451-487.

Havrylov, S., Ichioka, F., Powell, K., Borthwick, E. B., Baranska, J., Maki, M. and Buchman, V. L. (2008). Adaptor protein ruk/CIN85 is associated with a subset of COPI-coated membranes of the golgi complex. *Traffic* **9,** 798-812.

Hentschel, D. M., Mengel, M., Boehme, L., Liebsch, F., Albertin, C., Bonventre, J. V., Haller, H. and Schiffer, M. (2007). Rapid screening of glomerular slit diaphragm integrity in larval zebrafish. *Am. J. Physiol. Renal Physiol.* **293,** 1746.

Holthofer, H., Ahola, H., Solin, M. L., Wang, S., Palmen, T., Luimula, P., Miettinen, A. and Kerjaschki, D. (1999). Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. *Am. J. Pathol.* **155,** 1681-1687.

Höltje, H., Sippl, W., Rognan, D. and Folkers, G. (2003). *Molecular Modeling*. Weinheim: Wiley-VCH.

Hori, H., Sasaoka, T., Ishihara, H., Wada, T., Murakami, S., Ishiki, M. and Kobayashi, M. (2002). Association of SH2-containing inositol phosphatase 2 with the insulin resistance of diabetic db/db mice. *Diabetes* **51,** 2387-2394.

Huber, T. B., Hartleben, B., Kim, J., Schmidts, M., Schermer, B., Keil, A., Egger, L., Lecha, R. L., Borner, C., Pavenstadt, H. et al. (2003). Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol. Cell. Biol.* **23,** 4917-4928.

Hudkins, K. L., Pichaiwong, W., Wietecha, T., Kowalewska, J., Banas, M. C., Spencer, M. W., Muhlfeld, A., Koelling, M., Pippin, J. W., Shankland, S. J. et al. (2010). BTBR ob/ob mutant mice model progressive diabetic nephropathy. *J. Am. Soc. Nephrol.* **21,** 1533-1542.

Hughes, S. K., Oudin, M. J., Tadros, J., Neil, J., Del Rosario, A., Joughin, B. A., Ritsma, L., Wyckoff, J., Vasile, E., Eddy, R. et al. (2015). PTP1B-dependent regulation of receptor tyrosine kinase signaling by the actin-binding protein mena. *Mol. Biol. Cell* **26,** 3867-3878.

Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R. et al. (2000). Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49,** 2063-2069.

Hyvonen, M. E., Dumont, V., Tienari, J., Lehtonen, E., Ustinov, J., Havana, M., Jalanko, H., Otonkoski, T., Miettinen, P. J. and Lehtonen, S. (2015). Early-onset diabetic E1-DN mice develop albuminuria and glomerular injury typical of diabetic nephropathy. *Biomed. Res. Int.* **2015,** 102969.

Hyvonen, M. E., Ihalmo, P., Forsblom, C., Thorn, L., Sandholm, N., Lehtonen, S. and Groop, P. H. (2012). INPPL1 is associated with the metabolic syndrome in men with type 1 diabetes, but not with diabetic nephropathy. *Diabet. Med.* **29,** 1589-1595.

Hyvonen, M. E., Ihalmo, P., Sandholm, N., Stavarachi, M., Forsblom, C., McKnight, A. J., Lajer, M., Maestroni, A., Lewis, G., Tarnow, L. et al. (2013). CD2AP is associated with end-stage renal disease in patients with type 1 diabetes. *Acta Diabetol.* **50,** 887-897.

Hyvonen, M. E., Saurus, P., Wasik, A., Heikkila, E., Havana, M., Trokovic, R., Saleem, M., Holthofer, H. and Lehtonen, S. (2010). Lipid phosphatase SHIP2 downregulates insulin signalling in podocytes. *Mol. Cell. Endocrinol.* **328,** 70-79.

Ichihara, Y., Fujimura, R., Tsuneki, H., Wada, T., Okamoto, K., Gouda, H., Hirono, S., Sugimoto, K., Matsuya, Y., Sasaoka, T. et al. (2013). Rational design and synthesis of 4-substituted 2-pyridin-2-ylamides with inhibitory effects on SH2 domain-containing inositol 5'-phosphatase 2 (SHIP2). *Eur. J. Med. Chem.* **62,** 649-660.

Inagi, R. (2010). Endoplasmic reticulum stress as a progression factor for kidney injury. *Curr. Opin. Pharmacol.* **10,** 156-165.

Ishida, S., Funakoshi, A., Miyasaka, K., Shimokata, H., Ando, F. and Takiguchi, S. (2006). Association of SH-2 containing inositol 5'-phosphatase 2 gene polymorphisms and hyperglycemia. *Pancreas* **33,** 63-67.

Ishihara, H., Sasaoka, T., Hori, H., Wada, T., Hirai, H., Haruta, T., Langlois, W. J. and Kobayashi, M. (1999). Molecular cloning of rat SH2-containing inositol phosphatase 2 (SHIP2) and its role in the regulation of insulin signaling. *Biochem. Biophys. Res. Commun.* **260,** 265-272.

Jeansson, M. and Haraldsson, B. (2006). Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *Am. J. Physiol. Renal Physiol.* **290,** 111.

Jensen, P. J., Gunter, L. B. and Carayannopoulos, M. O. (2010). Akt2 modulates glucose availability and downstream apoptotic pathways during development. *J. Biol. Chem.* **285,** 17673-17680.

Jones, N., Blasutig, I. M., Eremina, V., Ruston, J. M., Bladt, F., Li, H., Huang, H., Larose, L., Li, S. S., Takano, T. et al. (2006). Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* **440,** 818-823.

Kagawa, S., Sasaoka, T., Yaguchi, S., Ishihara, H., Tsuneki, H., Murakami, S., Fukui, K., Wada, T., Kobayashi, S., Kimura, I. et al. (2005). Impact of SRC homology 2-containing inositol 5'-phosphatase 2 gene polymorphisms detected in a japanese population on insulin signaling. *J. Clin. Endocrinol. Metab.* **90,** 2911-2919.

Kagawa, S., Soeda, Y., Ishihara, H., Oya, T., Sasahara, M., Yaguchi, S., Oshita, R., Wada, T., Tsuneki, H. and Sasaoka, T. (2008). Impact of transgenic overexpression of SH2-containing inositol 5'-phosphatase 2 on glucose metabolism and insulin signaling in mice. *Endocrinology* **149,** 642-650.

Kahn, B. B., Rosen, A. S., Bak, J. F., Andersen, P. H., Damsbo, P., Lund, S. and Pedersen, O. (1992). Expression of GLUT1 and GLUT4 glucose transporters in skeletal muscle of humans with insulin-dependent diabetes mellitus: Regulatory effects of metabolic factors. *J. Clin. Endocrinol. Metab.* **74,** 1101-1109.

Kaisaki, P. J., Delepine, M., Woon, P. Y., Sebag-Montefiore, L., Wilder, S. P., Menzel, S., Vionnet, N., Marion, E., Riveline, J. P., Charpentier, G. et al. (2004). Polymorphisms in type II SH2 domain-containing inositol 5-phosphatase (INPPL1, SHIP2) are associated with physiological abnormalities of the metabolic syndrome. *Diabetes* **53,** 1900-1904.

Kandror, K. V. and Pilch, P. F. (2011). The sugar is sIRVed: Sorting Glut4 and its fellow travelers. *Traffic* **12,** 665-671.

Kang, J., Dai, X. S., Yu, T. B., Wen, B. and Yang, Z. W. (2005). Glycogen accumulation in renal tubules, a key morphological change in the diabetic rat kidney. *Acta Diabetol.* **42,** 110-116.

Kanzaki, M., Furukawa, M., Raab, W. and Pessin, J. E. (2004). Phosphatidylinositol 4,5-bisphosphate regulates adipocyte actin dynamics and GLUT4 vesicle recycling. *J. Biol. Chem.* **279**, 30622-30633.

Katz, E. B., Stenbit, A. E., Hatton, K., DePinho, R. and Charron, M. J. (1995). Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4. *Nature* **377,** 151-155.

Kawakami, T., Gomez, I. G., Ren, S., Hudkins, K., Roach, A., Alpers, C. E., Shankland, S. J., D'Agati, V. D. and Duffield, J. S. (2015). Deficient autophagy results in mitochondrial dysfunction and FSGS. *J. Am. Soc. Nephrol.* **26,** 1040-1052.

Kawano, K., Hirashima, T., Mori, S. and Natori, T. (1994). OLETF (otsuka long-evans tokushima fatty) rat: A new NIDDM rat strain. *Diabetes Res. Clin. Pract.* **24 Suppl,** 317.

Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R. et al. (1998). Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol. Cell* **1,** 575-582.

Kim, J. J., Li, J. J., Jung, D. S., Kwak, S. J., Ryu, D. R., Yoo, T. H., Han, S. H., Choi, H. Y., Kim, H. J., Han, D. S. et al. (2007). Differential expression of nephrin according to glomerular size in early diabetic kidney disease. *J. Am. Soc. Nephrol.* **18,** 2303-2310.

Kim, J. M., Wu, H., Green, G., Winkler, C. A., Kopp, J. B., Miner, J. H., Unanue, E. R. and Shaw, A. S. (2003). CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* **300,** 1298-1300.

Kim, J., Shon, E., Kim, C. S. and Kim, J. S. (2012). Renal podocyte injury in a rat model of type 2 diabetes is prevented by metformin. *Exp. Diabetes Res.* **2012,** 210821.

Kirsch, K. H., Georgescu, M. M., Ishimaru, S. and Hanafusa, H. (1999). CMS: An adapter molecule involved in cytoskeletal rearrangements. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6211-6216.

Klip, A., Sun, Y., Chiu, T. T. and Foley, K. P. (2014). Signal transduction meets vesicle traffic: The software and hardware of GLUT4 translocation. *Am. J. Physiol. Cell. Physiol.* **306,** 879.

Kobayashi, S., Sawano, A., Nojima, Y., Shibuya, M. and Maru, Y. (2004). The c-cbl/CD2AP complex regulates VEGF-induced endocytosis and degradation of flt-1 (VEGFR-1). *FASEB J.* **18,** 929-931.

Kristensen, J. M., Treebak, J. T., Schjerling, P., Goodyear, L. and Wojtaszewski, J. F. (2014). Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. *Am. J. Physiol. Endocrinol. Metab.* **306,** 1099.

Kriz, W., Gretz, N. and Lemley, K. V. (1998). Progression of glomerular diseases: Is the podocyte the culprit? *Kidney Int.* **54,** 687-697.

Kusturica, J., Kulo Cesic, A., Gusic, E., Maleskic, S., Rakanovic-Todic, M. and Secic, D. (2017). Metformin use associated with lower risk of cancer in patients with diabetes mellitus type 2. *Med. Glas. (Zenica)* **14,** 176-181.

Langer, S., Kreutz, R. and Eisenreich, A. (2016). Metformin modulates apoptosis and cell signaling of human podocytes under high glucose conditions. *J. Nephrol.* **29,** 765-773.

Langham, R. G., Kelly, D. J., Cox, A. J., Thomson, N. M., Holthofer, H., Zaoui, P., Pinel, N., Cordonnier, D. J. and Gilbert, R. E. (2002). Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: Effects of angiotensin converting enzyme inhibition. *Diabetologia* **45,** 1572-1576.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M. and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* **270,** 12953-12956.

Lehtonen, S., Ora, A., Olkkonen, V. M., Geng, L., Zerial, M., Somlo, S. and Lehtonen, E. (2000). In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2. *J. Biol. Chem.* **275,** 32888-32893.

Lehtonen, S., Tienari, J., Londesborough, A., Pirvola, U., Ora, A., Reima, I. and Lehtonen, E. (2008). CD2-associated protein is widely expressed and differentially regulated during embryonic development. *Differentiation* **76,** 506-517.

Lehtonen, S., Zhao, F. and Lehtonen, E. (2002). CD2-associated protein directly interacts with the actin cytoskeleton. *Am. J. Physiol. Renal Physiol.* **283,** 734.

Lewko, B. and Stepinski, J. (2009). Hyperglycemia and mechanical stress: Targeting the renal podocyte. *J. Cell. Physiol.* **221,** 288-295.

Li, C., Ruotsalainen, V., Tryggvason, K., Shaw, A. S. and Miner, J. H. (2000). CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am. J. Physiol. Renal Physiol.* **279,** 785.

Li, L. V. and Kandror, K. V. (2005). Golgi-localized, gamma-ear-containing, arf-binding protein adaptors mediate insulin-responsive

trafficking of glucose transporter 4 in 3T3-L1 adipocytes. *Mol. Endocrinol.* **19,** 2145-2153.

Like, A. A., Lavine, R. L., Poffenbarger, P. L. and Chick, W. L. (1972). Studies in the diabetic mutant mouse. VI. evolution of glomerular lesions and associated proteinuria. *Am. J. Pathol.* **66,** 193-224.

Lincova, E., Hampl, A., Pernicova, Z., Starsichova, A., Krcmar, P., Machala, M., Kozubik, A. and Soucek, K. (2009). Multiple defects in negative regulation of the PKB/akt pathway sensitise human cancer cells to the antiproliferative effect of non-steroidal anti-inflammatory drugs. *Biochem. Pharmacol.* **78,** 561-572.

Liu, A. and Coleman, S. P. (2009). Determination of metformin in human plasma using hydrophilic interaction liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci.* **877,** 3695-3700.

Liu, P., Begley, M., Michowski, W., Inuzuka, H., Ginzberg, M., Gao, D., Tsou, P., Gan, W., Papa, A., Kim, B. M. et al. (2014). Cell-cycle-regulated activation of akt kinase by phosphorylation at its carboxyl terminus. *Nature* **508,** 541-545.

Lynch, D. K., Winata, S. C., Lyons, R. J., Hughes, W. E., Lehrbach, G. M., Wasinger, V., Corthals, G., Cordwell, S. and Daly, R. J. (2003). A cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. *J. Biol. Chem.* **278,** 21805-21813.

Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X. M., Braddock, D. T., Albright, R. A., Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J. et al. (2014). Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* **510,** 542-546.

Maianu, L., Keller, S. R. and Garvey, W. T. (2001). Adipocytes exhibit abnormal subcellular distribution and translocation of vesicles containing glucose transporter 4 and insulin-regulated aminopeptidase in type 2 diabetes mellitus: Implications regarding defects in vesicle trafficking. *J. Clin. Endocrinol. Metab.* **86,** 5450-5456.

Mallipattu, S. K., Gallagher, E. J., LeRoith, D., Liu, R., Mehrotra, A., Horne, S. J., Chuang, P. Y., Yang, V. W. and He, J. C. (2014). Diabetic nephropathy in a nonobese mouse model of type 2 diabetes mellitus. *Am. J. Physiol. Renal Physiol.* **306,** 1008.

Marshall, C. B., Pippin, J. W., Krofft, R. D. and Shankland, S. J. (2006). Puromycin aminonucleoside induces oxidant-dependent DNA damage in podocytes in vitro and in vivo. *Kidney Int.* **70,** 1962-1973.

Martin, B. C., Warram, J. H., Krolewski, A. S., Bergman, R. N., Soeldner, J. S. and Kahn, C. R. (1992). Role of glucose and insulin resistance in development of type 2 diabetes mellitus: Results of a 25-year follow-up study. *Lancet* **340,** 925-929.

Martin, O. J., Lee, A. and McGraw, T. E. (2006). GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *J. Biol. Chem.* **281,** 484-490.

Mauer, S. M., Steffes, M. W., Ellis, E. N., Sutherland, D. E., Brown, D. M. and Goetz, F. C. (1984). Structural-functional relationships in diabetic nephropathy. *J. Clin. Invest.* **74,** 1143-1155.

Miele, C., Riboulet, A., Maitan, M. A., Oriente, F., Romano, C., Formisano, P., Giudicelli, J., Beguinot, F. and Van Obberghen, E. (2003). Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism. *J. Biol. Chem.* **278,** 47376-47387.

Mills, S. J., Persson, C., Cozier, G., Thomas, M. P., Tresaugues, L., Erneux, C., Riley, A. M., Nordlund, P. and Potter, B. V. (2012). A synthetic polyphosphoinositide headgroup surrogate in complex with SHIP2 provides a rationale for drug discovery. *ACS Chem. Biol.* **7,** 822-828.

Mima, A., Ohshiro, Y., Kitada, M., Matsumoto, M., Geraldes, P., Li, C., Li, Q., White, G. S., Cahill, C., Rask-Madsen, C. et al. (2011). Glomerular-specific protein kinase C-beta-induced insulin receptor substrate-1 dysfunction and insulin resistance in rat models of diabetes and obesity. *Kidney Int.* **79,** 883-896.

Minassian, C., Tarpin, S. and Mithieux, G. (1998). Role of glucose-6 phosphatase, glucokinase, and glucose-6 phosphate in liver insulin resistance and its correction by metformin. *Biochem. Pharmacol.* **55,** 1213-1219.

Miyazaki, Y., Cersosimo, E., Triplitt, C. and DeFronzo, R. A. (2007). Rosiglitazone decreases albuminuria in type 2 diabetic patients. *Kidney Int.* **72,** 1367-1373.

Mogensen, C. E. and Christensen, C. K. (1984). Predicting diabetic nephropathy in insulin-dependent patients. *N. Engl. J. Med.* **311,** 89-93.

Mundel, P. and Shankland, S. J. (2002). Podocyte biology and response to injury. *J. Am. Soc. Nephrol.* **13,** 3005-3015.

Najafian, B., Fogo, A. B., Lusco, M. A. and Alpers, C. E. (2015). AJKD atlas of renal pathology: Diabetic nephropathy. *Am. J. Kidney Dis.* **66,** 37.

Nakatsu, F., Perera, R. M., Lucast, L., Zoncu, R., Domin, J., Gertler, F. B., Toomre, D. and De Camilli, P. (2010). The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. *J. Cell Biol.* **190,** 307-315.

Nielsen, M. S., Madsen, P., Christensen, E. I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R. and Petersen, C. M. (2001). The sortilin cytoplasmic tail conveys golgi-endosome transport and binds the VHS domain of the GGA2 sorting protein. *EMBO J.* **20,** 2180-2190.

Nonogaki, K., Fuller, G. M., Fuentes, N. L., Moser, A. H., Staprans, I., Grunfeld, C. and Feingold, K. R. (1995). Interleukin-6 stimulates hepatic triglyceride secretion in rats. *Endocrinology* **136,** 2143-2149.

Ohno, T., Shimizu, M., Shirakami, Y., Baba, A., Kochi, T., Kubota, M., Tsurumi, H., Tanaka, T. and Moriwaki, H. (2015). Metformin suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-+Leprdb/+Leprdb mice. *PLoS One* **10,** e0124081.

Orchard, T. J., Chang, Y. F., Ferrell, R. E., Petro, N. and Ellis, D. E. (2002). Nephropathy in type 1 diabetes: A manifestation of insulin resistance and multiple genetic susceptibilities? further evidence from the pittsburgh epidemiology of diabetes complication study. *Kidney Int.* **62,** 963-970.

Pagtalunan, M. E., Miller, P. L., Jumping-Eagle, S., Nelson, R. G., Myers, B. D., Rennke, H. G., Coplon, N. S., Sun, L. and Meyer, T. W. (1997). Podocyte loss and progressive glomerular injury in type II diabetes. *J. Clin. Invest.* **99,** 342-348.

Palaniappan, L., Carnethon, M. and Fortmann, S. P. (2003). Association between microalbuminuria and the metabolic syndrome: NHANES III. *Am. J. Hypertens.* **16,** 952-958.

Parvanova, A. I., Trevisan, R., Iliev, I. P., Dimitrov, B. D., Vedovato, M., Tiengo, A., Remuzzi, G. and Ruggenenti, P. (2006). Insulin

resistance and microalbuminuria: A cross-sectional, case-control study of 158 patients with type 2 diabetes and different degrees of urinary albumin excretion. *Diabetes* **55,** 1456-1462.

Paz, K., Hemi, R., LeRoith, D., Karasik, A., Elhanany, E., Kanety, H. and Zick, Y. (1997). A molecular basis for insulin resistance. elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J. Biol. Chem.* **272,** 29911-29918.

Pechstein, A., Gerth, F., Milosevic, I., Japel, M., Eichhorn-Grunig, M., Vorontsova, O., Bacetic, J., Maritzen, T., Shupliakov, O., Freund, C. et al. (2015). Vesicle uncoating regulated by SH3-SH3 domain-mediated complex formation between endophilin and intersectin at synapses. *EMBO Rep.* **16,** 232-239.

Perkins, B. A., Ficociello, L. H., Silva, K. H., Finkelstein, D. M., Warram, J. H. and Krolewski, A. S. (2003). Regression of microalbuminuria in type 1 diabetes. *N. Engl. J. Med.* **348,** 2285-2293.

Pesesse, X., Deleu, S., De Smedt, F., Drayer, L. and Erneux, C. (1997). Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem. Biophys. Res. Commun.* **239,** 697-700.

Pesesse, X., Moreau, C., Drayer, A. L., Woscholski, R., Parker, P. and Erneux, C. (1998). The SH2 domain containing inositol 5-phosphatase SHIP2 displays phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate 5-phosphatase activity. *FEBS Lett.* **437,** 301-303.

Pessin, J. E. and Saltiel, A. R. (2000). Signaling pathways in insulin action: Molecular targets of insulin resistance. *J. Clin. Invest.* **106,** 165-169.

Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J. R. and Pedersen, B. K. (2005). Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of akt substrate 160 phosphorylation. *Diabetes* **54,** 2939-2945.

Plomgaard, P., Fischer, C. P., Ibfelt, T., Pedersen, B. K. and van Hall, G. (2008). Tumor necrosis factor-alpha modulates human in vivo lipolysis. *J. Clin. Endocrinol. Metab.* **93,** 543-549.

Potashnik, R., Bloch-Damti, A., Bashan, N. and Rudich, A. (2003). IRS1 degradation and increased serine phosphorylation cannot predict the

degree of metabolic insulin resistance induced by oxidative stress. *Diabetologia* **46,** 639-648.

Poussu, A. M., Virtanen, I., Autio-Harmainen, H. and Lehto, V. P. (2001). Podocyte-specific expression of a novel trans-golgi protein vear in human kidney. *Kidney Int.* **60,** 626-634.

Prasad, N. K. (2009). SHIP2 phosphoinositol phosphatase positively regulates EGFR-akt pathway, CXCR4 expression, and cell migration in MDA-MB-231 breast cancer cells. *Int. J. Oncol.* **34,** 97-105.

Prasad, N. K. and Decker, S. J. (2005). SH2-containing 5'-inositol phosphatase, SHIP2, regulates cytoskeleton organization and ligand-dependent down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* **280,** 13129-13136.

Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T. and Bos, J. L. (1993). Insulin-induced phosphorylation of the 46- and 52-kDa shc proteins. *J. Biol. Chem.* **268,** 5748-5753.

Puertollano, R., Randazzo, P. A., Presley, J. F., Hartnell, L. M. and Bonifacino, J. S. (2001). The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell* **105,** 93-102.

Raij, L., Tian, R., Wong, J. S., He, J. C. and Campbell, K. N. (2016). Podocyte injury: The role of proteinuria, urinary plasminogen, and oxidative stress. *Am. J. Physiol. Renal Physiol.* **311,** F1317.

Reutens, A. T. and Atkins, R. C. (2011). Epidemiology of diabetic nephropathy. *Contrib. Nephrol.* **170,** 1-7.

Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W. and Shulman, G. I. (1996). Mechanism of free fatty acid-induced insulin resistance in humans. *J. Clin. Invest.* **97,** 2859-2865.

Rodewald, R. and Karnovsky, M. J. (1974). Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J. Cell Biol.* **60,** 423-433.

Rogacka, D., Audzeyenka, I., Rychlowski, M., Rachubik, P., Szrejder, M., Angielski, S. and Piwkowska, A. (2017). Metformin overcomes high glucose-induced insulin resistance of podocytes by pleiotropic effects on SIRT1 and AMPK. *Biochim. Biophys. Acta* **1864,** 115-125.

Rogacka, D., Piwkowska, A., Audzeyenka, I., Angielski, S. and Jankowski, M. (2014). Involvement of the AMPK-PTEN pathway in insulin

resistance induced by high glucose in cultured rat podocytes. *Int. J. Biochem. Cell Biol.* **51,** 120-130.

Rogacka, D., Piwkowska, A., Audzeyenka, I., Angielski, S. and Jankowski, M. (2016). SIRT1-AMPK crosstalk is involved in high glucose-dependent impairment of insulin responsiveness in primary rat podocytes. *Exp. Cell Res.* **349,** 328-338.

Ruotsalainen, V., Ljungberg, P., Wartiovaara, J., Lenkkeri, U., Kestila, M., Jalanko, H., Holmberg, C. and Tryggvason, K. (1999). Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7962-7967.

Saleem, M. A., O'Hare, M. J., Reiser, J., Coward, R. J., Inward, C. D., Farren, T., Xing, C. Y., Ni, L., Mathieson, P. W. and Mundel, P. (2002). A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J. Am. Soc. Nephrol.* **13,** 630-638.

Saltiel, A. R. and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799-806.

Sanwal, V., Pandya, M., Bhaskaran, M., Franki, N., Reddy, K., Ding, G., Kapasi, A., Valderrama, E. and Singhal, P. C. (2001). Puromycin aminonucleoside induces glomerular epithelial cell apoptosis. *Exp. Mol. Pathol.* **70,** 54-64.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005). Phosphorylation and regulation of akt/PKB by the rictor-mTOR complex. *Science* **307,** 1098-1101.

Sasaoka, T., Wada, T. and Tsuneki, H. (2006). Lipid phosphatases as a possible therapeutic target in cases of type 2 diabetes and obesity. *Pharmacol. Ther.* **112,** 799-809.

Saurus, P., Kuusela, S., Dumont, V., Lehtonen, E., Fogarty, C. L., Lassenius, M. I., Forsblom, C., Lehto, M., Saleem, M. A., Groop, P. H. et al. (2016). Cyclin-dependent kinase 2 protects podocytes from apoptosis. *Sci. Rep.* **6,** 21664.

Saurus, P., Kuusela, S., Lehtonen, E., Hyvonen, M. E., Ristola, M., Fogarty, C. L., Tienari, J., Lassenius, M. I., Forsblom, C., Lehto, M. et al. (2015). Podocyte apoptosis is prevented by blocking the toll-like receptor pathway. *Cell. Death Dis.* **6,** e1752.

Schiffer, M., Bitzer, M., Roberts, I. S., Kopp, J. B., ten Dijke, P., Mundel, P. and Bottinger, E. P. (2001). Apoptosis in podocytes induced by TGF-beta and Smad7. *J. Clin. Invest.* **108,** 807-816.

Schiffer, M., Mundel, P., Shaw, A. S. and Bottinger, E. P. (2004). A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. *J. Biol. Chem.* **279,** 37004-37012.

Schiffer, M., Susztak, K., Ranalletta, M., Raff, A. C., Bottinger, E. P. and Charron, M. J. (2005). Localization of the GLUT8 glucose transporter in murine kidney and regulation in vivo in nondiabetic and diabetic conditions. *Am. J. Physiol. Renal Physiol.* **289,** 186.

Sharma, V. P., Eddy, R., Entenberg, D., Kai, M., Gertler, F. B. and Condeelis, J. (2013). Tks5 and SHIP2 regulate invadopodium maturation, but not initiation, in breast carcinoma cells. *Curr. Biol.* **23,** 2079-2089.

Shi, J. and Kandror, K. V. (2005). Sortilin is essential and sufficient for the formation of Glut4 storage vesicles in 3T3-L1 adipocytes. *Dev. Cell.* **9,** 99-108.

Shi, J. and Kandror, K. V. (2007). The luminal Vps10p domain of sortilin plays the predominant role in targeting to insulin-responsive Glut4-containing vesicles. *J. Biol. Chem.* **282,** 9008-9016.

Shih, N. Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M. L., Kanagawa, O., Miner, J. H. and Shaw, A. S. (1999). Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* **286,** 312-315.

Sleeman, M. W., Wortley, K. E., Lai, K. M., Gowen, L. C., Kintner, J., Kline, W. O., Garcia, K., Stitt, T. N., Yancopoulos, G. D., Wiegand, S. J. et al. (2005). Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nat. Med.* **11,** 199-205.

Soeda, Y., Tsuneki, H., Muranaka, H., Mori, N., Hosoh, S., Ichihara, Y., Kagawa, S., Wang, X., Toyooka, N., Takamura, Y. et al. (2010). The inositol phosphatase SHIP2 negatively regulates insulin/IGF-I actions implicated in neuroprotection and memory function in mouse brain. *Mol. Endocrinol.* **24,** 1965-1977.

Sperandio, S., Poksay, K., de Belle, I., Lafuente, M. J., Liu, B., Nasir, J. and Bredesen, D. E. (2004). Paraptosis: Mediation by MAP kinases and inhibition by AIP-1/alix. *Cell Death Differ.* **11,** 1066-1075.

St John, P. L. and Abrahamson, D. R. (2001). Glomerular endothelial cells and podocytes jointly synthesize laminin-1 and -11 chains. *Kidney Int.* **60,** 1037-1046.

Stepien, M., Stepien, A., Wlazel, R. N., Paradowski, M., Rizzo, M., Banach, M. and Rysz, J. (2014). Predictors of insulin resistance in patients with obesity: A pilot study. *Angiology* **65,** 22-30.

Susztak, K., Raff, A. C., Schiffer, M. and Bottinger, E. P. (2006). Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* **55,** 225-233.

Suwa, A., Kurama, T. and Shimokawa, T. (2010a). SHIP2 and its involvement in various diseases. *Expert Opin. Ther. Targets* **14,** 727-737.

Suwa, A., Kurama, T., Yamamoto, T., Sawada, A., Shimokawa, T. and Aramori, I. (2010b). Glucose metabolism activation by SHIP2 inhibitors via up-regulation of GLUT1 gene in L6 myotubes. *Eur. J. Pharmacol.* **642,** 177-182.

Suwa, A., Yamamoto, T., Sawada, A., Minoura, K., Hosogai, N., Tahara, A., Kurama, T., Shimokawa, T. and Aramori, I. (2009). Discovery and functional characterization of a novel small molecule inhibitor of the intracellular phosphatase, SHIP2. *Br. J. Pharmacol.* **158,** 879-887.

Tejada, T., Catanuto, P., Ijaz, A., Santos, J. V., Xia, X., Sanchez, P., Sanabria, N., Lenz, O., Elliot, S. J. and Fornoni, A. (2008). Failure to phosphorylate AKT in podocytes from mice with early diabetic nephropathy promotes cell death. *Kidney Int.* **73,** 1385-1393.

Tervaert, T. W., Mooyaart, A. L., Amann, K., Cohen, A. H., Cook, H. T., Drachenberg, C. B., Ferrario, F., Fogo, A. B., Haas, M., de Heer, E. et al. (2010). Pathologic classification of diabetic nephropathy. *J. Am. Soc. Nephrol.* **21,** 556-563.

Tian, W., Zhang, Z. and Cohen, D. M. (2000). MAPK signaling and the kidney. *Am. J. Physiol. Renal Physiol.* **279,** 593.

Tolvanen, T. A. (2008). Ligand based virtual screening methods.

Tossidou, I., Kardinal, C., Peters, I., Kriz, W., Shaw, A., Dikic, I., Tkachuk, S., Dumler, I., Haller, H. and Schiffer, M. (2007). CD2AP/CIN85 balance determines receptor tyrosine kinase signaling response in podocytes. *J. Biol. Chem.* **282,** 7457-7464.

Toyoda, M., Najafian, B., Kim, Y., Caramori, M. L. and Mauer, M. (2007). Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type 1 diabetic nephropathy. *Diabetes* **56,** 2155-2160.

Tresaugues, L., Silvander, C., Flodin, S., Welin, M., Nyman, T., Graslund, S., Hammarstrom, M., Berglund, H. and Nordlund, P. (2014). Structural basis for phosphoinositide substrate recognition, catalysis, and membrane interactions in human inositol polyphosphate 5-phosphatases. *Structure* **22,** 744-755.

Turban, S., Stretton, C., Drouin, O., Green, C. J., Watson, M. L., Gray, A., Ross, F., Lantier, L., Viollet, B., Hardie, D. G. et al. (2012). Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. *J. Biol. Chem.* **287,** 20088-20099.

Vallon, V. (2011). The proximal tubule in the pathophysiology of the diabetic kidney. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300,** 1009.

Vandeput, F., Combettes, L., Mills, S. J., Backers, K., Wohlkonig, A., Parys, J. B., De Smedt, H., Missiaen, L., Dupont, G., Potter, B. V. et al. (2007). Biphenyl 2,3',4,5',6-pentakisphosphate, a novel inositol polyphosphate surrogate, modulates Ca2+ responses in rat hepatocytes. *FASEB J.* **21,** 1481-1491.

Verzola, D., Gandolfo, M. T., Ferrario, F., Rastaldi, M. P., Villaggio, B., Gianiorio, F., Giannoni, M., Rimoldi, L., Lauria, F., Miji, M. et al. (2007). Apoptosis in the kidneys of patients with type II diabetic nephropathy. *Kidney Int.* **72,** 1262-1272.

Viberti, G. C., Hill, R. D., Jarrett, R. J., Argyropoulos, A., Mahmud, U. and Keen, H. (1982). Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* **1,** 1430-1432.

Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M. and Andreelli, F. (2012). Cellular and molecular mechanisms of metformin: An overview. *Clin. Sci. (Lond)* **122,** 253-270.

Wada, T., Sasaoka, T., Funaki, M., Hori, H., Murakami, S., Ishiki, M., Haruta, T., Asano, T., Ogawa, W., Ishihara, H. et al. (2001). Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5'-phosphatase catalytic activity. *Mol. Cell. Biol.* **21,** 1633-1646.

Wang, J., Takeuchi, T., Tanaka, S., Kubo, S. K., Kayo, T., Lu, D., Takata, K., Koizumi, A. and Izumi, T. (1999). A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the mody mouse. *J. Clin. Invest.* **103,** 27-37.

Wang, Z., Wei, X., Zhang, Y., Ma, X., Li, B., Zhang, S., Du, P., Zhang, X. and Yi, F. (2009). NADPH oxidase-derived ROS contributes to upregulation of TRPC6 expression in puromycin aminonucleoside-induced podocyte injury. *Cell. Physiol. Biochem.* **24,** 619-626.

Wasik, A. A., Polianskyte-Prause, Z., Dong, M. Q., Shaw, A. S., Yates, J. R., Farquhar, M. G. and Lehtonen, S. (2012). Septin 7 forms a complex with CD2AP and nephrin and regulates glucose transporter trafficking. *Mol. Biol. Cell* **23,** 3370-3379.

Waters, S. B., D'Auria, M., Martin, S. S., Nguyen, C., Kozma, L. M. and Luskey, K. L. (1997). The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes. *J. Biol. Chem.* **272,** 23323-23327.

Weil, E. J., Lemley, K. V., Mason, C. C., Yee, B., Jones, L. I., Blouch, K., Lovato, T., Richardson, M., Myers, B. D. and Nelson, R. G. (2012). Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. *Kidney Int.* **82,** 1010-1017.

Welsch, T., Endlich, N., Gokce, G., Doroshenko, E., Simpson, J. C., Kriz, W., Shaw, A. S. and Endlich, K. (2005). Association of CD2AP with dynamic actin on vesicles in podocytes. *Am. J. Physiol. Renal Physiol.* **289,** 1134.

Welsh, G. I., Hale, L. J., Eremina, V., Jeansson, M., Maezawa, Y., Lennon, R., Pons, D. A., Owen, R. J., Satchell, S. C., Miles, M. J. et al. (2010). Insulin signaling to the glomerular podocyte is critical for normal kidney function. *Cell. Metab.* **12,** 329-340.

Wharram, B. L., Goyal, M., Wiggins, J. E., Sanden, S. K., Hussain, S., Filipiak, W. E., Saunders, T. L., Dysko, R. C., Kohno, K., Holzman, L. B. et al. (2005). Podocyte depletion causes glomerulosclerosis: Diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J. Am. Soc. Nephrol.* **16,** 2941-2952.

White, K. E. and Bilous, R. W. (2000). Type 2 diabetic patients with nephropathy show structural-functional relationships that are similar to type 1 disease. *J. Am. Soc. Nephrol.* **11,** 1667-1673.

Wiernsperger, N. F. (1999). Membrane physiology as a basis for the cellular effects of metformin in insulin resistance and diabetes. *Diabetes Metab.* **25,** 110-127.

Wiggins, J. E., Goyal, M., Sanden, S. K., Wharram, B. L., Shedden, K. A., Misek, D. E., Kuick, R. D. and Wiggins, R. C. (2005). Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: Prevention by calorie restriction. *J. Am. Soc. Nephrol.* **16,** 2953-2966.

Wu, Y., Dong, J., Yuan, L., Liang, C., Ren, K., Zhang, W., Fang, F. and Shen, J. (2008). Nephrin and podocin loss is prevented by mycophenolate mofetil in early experimental diabetic nephropathy. *Cytokine* **44,** 85-91.

Wullschleger, S., Wasserman, D. H., Gray, A., Sakamoto, K. and Alessi, D. R. (2011). Role of TAPP1 and TAPP2 adaptor binding to PtdIns(3,4)P2 in regulating insulin sensitivity defined by knock-in analysis. *Biochem. J.* **434,** 265-274.

Xiong, W., Jordens, I., Gonzalez, E. and McGraw, T. E. (2010). GLUT4 is sorted to vesicles whose accumulation beneath and insertion into the plasma membrane are differentially regulated by insulin and selectively affected by insulin resistance. *Mol. Biol. Cell* **21,** 1375-1386.

Yaddanapudi, S., Altintas, M. M., Kistler, A. D., Fernandez, I., Moller, C. C., Wei, C., Peev, V., Flesche, J. B., Forst, A. L., Li, J. et al. (2011). CD2AP in mouse and human podocytes controls a proteolytic program that regulates cytoskeletal structure and cellular survival. *J. Clin. Invest.* **121,** 3965-3980.

Yan, K., Ito, N., Nakajo, A., Kurayama, R., Fukuhara, D., Nishibori, Y., Kudo, A., Akimoto, Y. and Takenaka, H. (2012). The struggle for energy in podocytes leads to nephrotic syndrome. *Cell. Cycle* **11,** 1504-1511.

Yang, J. and Holman, G. D. (2006). Long-term metformin treatment stimulates cardiomyocyte glucose transport through an AMP-activated protein kinase-dependent reduction in GLUT4 endocytosis. *Endocrinology* **147,** 2728-2736.

Ye, Y., Ge, Y. M., Xiao, M. M., Guo, L. M., Li, Q., Hao, J. Q., Da, J., Hu, W. L., Zhang, X. D., Xu, J. et al. (2016). Suppression of SHIP2 contributes to tumorigenesis and proliferation of gastric cancer cells via activation of akt. *J. Gastroenterol.* **51,** 230-240.

Yip, J., Mattock, M. B., Morocutti, A., Sethi, M., Trevisan, R. and Viberti, G. (1993). Insulin resistance in insulin-dependent diabetic patients with microalbuminuria. *Lancet* **342,** 883-887.

Yoshinaga, S., Ohkubo, T., Sasaki, S., Nuriya, M., Ogawa, Y., Yasui, M., Tabata, H. and Nakajima, K. (2012). A phosphatidylinositol lipids system, lamellipodin, and ena/VASP regulate dynamic morphology of multipolar migrating cells in the developing cerebral cortex. *J. Neurosci.* **32,** 11643-11656.

Yuan, L., Ziegler, R. and Hamann, A. (2002). Inhibition of phosphoenolpyruvate carboxykinase gene expression by metformin in cultured hepatocytes. *Chin. Med. J. (Engl)* **115,** 1843-1848.

Zhai, L., Gu, J., Yang, D., Hu, W., Wang, W. and Ye, S. (2017). Metformin ameliorates podocyte damage by restoring renal tissue nephrin expression in type 2 diabetic rats. *J. Diabetes* **9,** 510-517.

Zhang, H., Schin, M., Saha, J., Burke, K., Holzman, L. B., Filipiak, W., Saunders, T., Xiang, M., Heilig, C. W. and Brosius, F. C. (2010). Podocyte-specific overexpression of GLUT1 surprisingly reduces mesangial matrix expansion in diabetic nephropathy in mice. *Am. J. Physiol. Renal Physiol.* **299,** 91.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N. et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* **108,** 1167-1174.

Zhuang, G., Hunter, S., Hwang, Y. and Chen, J. (2007). Regulation of EphA2 receptor endocytosis by SHIP2 lipid phosphatase via phosphatidylinositol 3-kinase-dependent Rac1 activation. *J. Biol. Chem.* **282,** 2683-2694.

Zimmet, P., Alberti, K. G. and Shaw, J. (2001). Global and societal implications of the diabetes epidemic. *Nature* **414,** 782-787.

Zucker, L. M. and Antoniades, H. N. (1972). Insulin and obesity in the zucker genetically obese rat "fatty". *Endocrinology* **90,** 1320-1330.