

REVIEW

Mitochondrial dysfunction and mitophagy: the beginning and end to diabetic nephropathy?

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Diabetic nephropathy (DN) is a progressive microvascular complication arising from diabetes. Within the kidney, the glomeruli, tubules, vessels and interstitium are disrupted, ultimately impairing renal function and leading to end-stage renal disease (ESRD). Current pharmacological therapies used in individuals with DN do not prevent the inevitable progression to ESRD; therefore, new targets of therapy are urgently required. Studies from animal models indicate that disturbances in mitochondrial homeostasis are central to the pathogenesis of DN. Since renal proximal tubule cells rely on oxidative phosphorylation to provide adequate ATP for tubular reabsorption, an impairment of mitochondrial bioenergetics can result in renal functional decline. Defects at the level of the electron transport chain have long been established in DN, promoting electron leakage and formation of superoxide radicals, mediating microinflammation and contributing to the renal lesion. More recent studies suggest that mitochondrial-associated proteins may be directly involved in the pathogenesis of tubulointerstitial fibrosis and glomerulosclerosis. An accumulation of fragmented mitochondria are found in the renal cortex in both humans and animals with DN, suggesting that in tandem with a shift in dynamics, mitochondrial clearance mechanisms may be impaired. The process of mitophagy is the selective targeting of damaged or dysfunctional mitochondria to autophagosomes for degradation through the autophagy pathway. The current review explores the concept that an impairment in the mitophagy system leads to the accelerated progression of renal pathology. A better understanding of the cellular and molecular events that govern mitophagy and dynamics in DN may lead to improved therapeutic strategies.

LINKED ARTICLES

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Abbreviations

$\Delta\Psi_m$, mitochondrial membrane potential; ACD, autophagic cell death; AIF, apoptosis-inducing factor; AKI, acute kidney injury; ANT, adenine nucleotide translocator; CKD, chronic kidney disease; CoQ, coenzyme Q; Cyp-D, cyclophilin D; cyt c, cytochrome c; DN, diabetic nephropathy; Drp1, dynamin-related protein 1; ECM, extracellular matrix; ER, endoplasmic reticulum; ESRD, end-stage renal disease; ETC, electron transport chain; FOXO3a, Forkhead box O3a; FUNDC1, FUN14 domain containing 1; GFR, glomerular filtration rate; Gpx, glutathione peroxidase; HDAC6, histone deacetylase 6; HIF-1 α , hypoxia-inducible factor 1 α ; HK2, hexokinase 2; IHG-1, induced-in-high-glucose-1; IMM, inner mitochondrial membrane; IMS, inter-membrane space; KHC, kinesin-1 heavy chain; LC3, light chain 3; LIR, LC3 interacting region; Mfn1/2, mitofusin 1/2; Miro, mitochondria Rho GTPase; MitoQ, mitoquinol; MOMP, mitochondrial outer membrane pore; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; mROS, mitochondrial reactive oxygen species; mtDNA, mitochondrial DNA; mTOR, mechanistic target of rapamycin; mTORC1/2, mechanistic target of rapamycin complex 1/2; MTS, mitochondria targeting sequence; NOX4, NADPH oxidase 4; Nrf2, nuclear factor (erythroid-derived 2)-like 2; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1; OXPHOS, oxidative phosphorylation; PGC1 α , peroxisome proliferator-activated receptor γ co-activator 1 α ; PINK1, PTEN-induced putative kinase 1; PTEC, proximal tubule epithelial cell; RAS, renin angiotensin system; ROS, reactive oxygen species; SGLT2, sodium glucose transporter 2; SQSTM1, sequestosome 1; STZ, streptozotocin; TIM, translocase of

the inner mitochondrial membrane; TIGAR, TP53-induced glycolysis and apoptosis regulator; TOM, translocase of the outer mitochondrial membrane; TPP⁺, triphenylalkylphosphonium cation; UBD, ubiquitin-binding domain; UPS, ubiquitin proteasome system; UVRAG, ultraviolet resistance-associated gene; VDAC1, voltage-dependent anion channel 1

Introduction

Diabetic nephropathy (DN) refers to chronic kidney disease (CKD) initiated by diabetes mellitus (2007). DN, which affects approximately 30% of patients with diabetes (Parving *et al.*, 2006; Thomas *et al.*, 2006; 2007; Nathan *et al.*, 2009), is the most common cause of end-stage renal disease (ESRD) in Western societies (Gilbertson *et al.*, 2005). Patients with DN carry a higher risk for co-morbidities such as cardiovascular disease (Borch-Johnsen and Kreiner, 1987), and indeed, renal dysfunction is a major predictor of all-cause mortality (Matsushita *et al.*, 2010). Not only does diabetes and its complications impose a substantial burden on the economy, but it also inflicts high costs on society, both in terms of reduced quality of life, and pain and suffering of people with diabetes (2013). As the prevalence of both type 1 and type 2 diabetes is increasing (Whiting *et al.*, 2011), these costs are set to escalate, making diabetes and its complications an urgent public health issue.

The natural history of DN is the development of persistent microalbuminuria (presence of albumin in the urine), which in some patients progresses to overt proteinuria followed by a gradual decline in glomerular filtration rate (GFR), eventually leading to renal failure (Parving *et al.*, 1981). However, not all patients fit the classical paradigm, as there is increasing evidence of renal impairment in the absence of albuminuria in some patients with DN (Perkins *et al.*, 2003; Hovind *et al.*, 2004; de Boer *et al.*, 2011). The presence and severity of nephropathy is associated with premature mortality due to cardiovascular causes (Ninomiya *et al.*, 2009; Whitman *et al.*, 2012). Changes in renal function are associated with ultrastructural changes involving all four renal compartments: glomeruli, tubules, interstitium and vessels. Within the glomerular and tubulointerstitial compartments, there is an accumulation of extracellular matrices (ECMs) (Raparia *et al.*, 2013). Thickening of the glomerular basement membrane (Parving *et al.*, 1992) is paralleled by thickening of the tubular basement membrane (Brito *et al.*, 1998). Other renal lesions include afferent and efferent arteriolar hyaline sclerosis (Mauer *et al.*, 1981). Progressive renal functional loss results with advanced disease, due to glomerular collapse and scarring (i.e. sclerosis), by capillary lumen obliteration resulting from mesangial expansion and by tubular atrophy (Steinke, 2009).

The Diabetes Control and Complications Trial, which examined whether long-term complications of diabetes could be prevented or delayed by implementing therapies aimed at achieving blood glucose levels as close as possible to the normal range (HbA_{1c} < 6.05%), revealed that intensive therapy reduced the risks for the development and progression of microalbuminuria (1993). This implies that hyperglycaemia is a fundamental cause of microvascular complications. However, more recent studies have confirmed that intensive treatment does not prevent loss of GFR or

progression to ESRD (Foundation, 2012). Hence, other variables are needed to explain why only a subset of patients with DN progress to ESRD.

Inhibition of the renin angiotensin system (RAS) is the major focus of the current clinical therapy. However, agents that target the RAS, such as ACE inhibitors or angiotensin receptor-1 antagonists, have been only partially successful as these compounds just delay, rather than prevent, the progression to ESRD (Brenner *et al.*, 2001; Bilous *et al.*, 2009; Mauer *et al.*, 2009). Thus, there is an urgent need to identify better targets to prevent ESRD.

The pathogenesis of DN is extremely complex as the kidney is not only composed of multiple cell populations, but is involved in several essential regulatory functions including, but not limited to, BP regulation, filtration of toxins, maintenance of acid–base balance, regulation of electrolytes, reabsorption of nutrients and hormone secretion (Brenner and Rector, 2008). Several key pathways of pathology thought to promote renal dysfunction leading to DN include an abnormally active RAS, overproduction of reactive oxygen species (ROS), increased glucose metabolite flux, advanced glycation end-products, endoplasmic reticulum (ER) stress and enhanced pro-inflammatory cytokine signalling (recently reviewed in Forbes and Cooper, 2013; Rask-Madsen and King, 2013). Increasing evidence indicates that the disruption of mitochondrial bioenergetics may be important in the development and progression of DN (Forbes *et al.*, 2008; Sivitz and Yorek, 2010; Sharma *et al.*, 2013). As the kidney relies on oxidative phosphorylation (OXPHOS) to provide the bulk requirements of ATP for tubular reabsorption (Soltoff, 1986), it is not surprising that mitochondrial homeostasis is strictly essential for an optimally functioning kidney. Indeed, in animal models of DN, it has been shown that mitochondrial ATP is depleted in the renal cortex in late diabetes, coinciding with glomerular and tubulointerstitial pathology (Tan *et al.*, 2010).

Mitochondrial dysfunction occurs following inhibition of OXPHOS, which results in decreased ATP production, loss of mitochondrial membrane potential ($\Delta\Psi_m$) and can ultimately lead to changes in cationic gradients (e.g. cytosolic Ca²⁺ concentrations), increased ROS from various sites of the electron transport chain (ETC) and, ultimately, redistribution of mitochondrial cell death proteins. Whereas much attention has been given to the molecular events that govern mitochondrial homeostasis in cancer (Wallace, 2012) and neurodegeneration, such as Parkinson's disease and Alzheimer's disease (Higgins *et al.*, 2010; Lezi and Swerdlow, 2012), limited investigations have been undertaken on these events in DN. The high energy demands of the brain and malignant tumours depend heavily on functional mitochondria. Similarly, cells in the kidney – particularly the mitochondria-rich proximal tubule epithelial cells (PTECs) – have high ATP requirements to drive tubular reabsorption (Harris *et al.*, 1981; Balaban and Mandel, 1988). Yet, in the context of DN, there has been

comparatively less research undertaken on mitochondrial biology.

Autophagy is the process responsible for recycling organelles and long-lived proteins to maintain cellular homeostasis. Autophagy can be stimulated in the face of cellular stress or starvation to meet increased energy demands or to remove toxic protein aggregates and damaged organelles (Yang and Klionsky, 2010a). Autophagy comes in three forms: micro-, macro- and chaperone-mediated autophagy. Macroautophagy (herein referred to as autophagy) is distinguished from other forms of autophagy, as it is the only process that requires the encapsulation of cellular debris in double membrane vesicles, known as autophagosomes. Autophagosomes transport their contents to lysosomes where fusion occurs, and the contents are transferred to the acidic organelle for degradation. The contents, once degraded, are recycled to generate new proteins and organelles. The removal of dysfunctional or redundant mitochondria from cells can occur by selective or non-selective autophagy. Targeted degradation of mitochondria by autophagic machinery is termed 'mitophagy' (Lemasters, 2005). The role of mitophagy in DN is of particular interest given that accumulations of damaged mitochondria have been observed in the kidney, raising the possibility that an impairment in the mitophagy system may be present.

The current review aims to focus on the role of impaired mitochondrial homeostasis in the aetiology of DN and to provide new insights into bioenergetic and morphological regulation of mitochondria in the kidney. In particular, the role of autophagy and mitophagy and the potential contribution to mitochondrial dysfunction in DN will be examined. Knowledge on mitochondria and autophagy gained from other fields of cell biology will be discussed, in addition to putative targets for therapeutic intervention in DN. As there has been increasing emphasis on the importance of mitochondria and autophagy in the development and progression of many other chronic diseases, particularly cancer and neurodegeneration, it is likely that these pathways could also be aberrant in the diabetic kidney.

Cause or consequence: mitochondrial dysfunction during DN

Hyperglycaemia is thought to promote oxidative stress during diabetic complications such as DN (Baynes, 1991; Brownlee, 2001). Oxidative stress can be directly toxic to cells, causing damage to DNA, proteins and lipids. However, the significance of excessive ROS to the pathology of DN remains to be fully determined, as it is not yet known whether oxidative stress has a primary role in the pathogenesis of complications (Baynes and Thorpe, 1999). ROS is produced from several sources in DN, with mitochondria considered one of the major sites (Forbes *et al.*, 2008). Mitochondrial dysfunction in DN may have far wider implications for the pathogenesis of the disease as it can result in a decline in ATP required to maintain cellular homeostasis, in particular to drive the Na^+/K^+ -ATPase in the proximal tubule to preserve the Na^+ gradient (Soltoff, 1986). Furthermore, end-stage mitochondrial dysfunction can lead to intrinsic cell

death. Ultimately, the question of whether mitochondrial dysfunction is the cause or consequence of DN becomes irrelevant. Without healthy mitochondria, cellular homeostasis within the proximal tubule will degenerate (Figure 1). Thus, therapeutic intervention of DN must incorporate strategies directed towards alleviating mitochondrial dysfunction (discussed later).

During hyperglycaemia, plasma glucose exceeds the filtration rate of the proximal tubule, resulting in glucose excretion in urine (Mather and Pollock, 2011). In the early onset of DN, an increased glucose load is thus placed on the apical sodium glucose transporter 2 (SGLT2), as glucose is transported from the tubular lumen into the PTECs against the glucose gradient (Figure 1A). SGLT2 relies on active basolateral Na^+/K^+ -ATPase pumps to remove Na^+ from the PTECs into the peritubular capillary, creating a downhill Na^+ gradient between the tubular lumen and the PTECs. The downhill Na^+ gradient allows co-transport of Na^+ and glucose into the proximal tubule from the tubular lumen (Vallon, 2011). Glucose is, in turn, passed from the PTECs into the peritubular capillary via the glucose uniporter GLUT2. This complex process is reliant on functional mitochondria to produce an ample pool of ATP to drive the Na^+/K^+ -ATPase pump (Figure 1A). Under the diabetic milieu, in order to maintain the glucose gradient, there is an increased demand for ATP, which, unless satisfied, will lead to PTEC impairment. Indeed, a decrease in the ATP pool is the initiating factor in the development of both lethal and sub-lethal renal PTEC injury, and the degree of ATP decline determines the severity of injury (Bonventre and Weinberg, 2003).

Mitochondria are required to increase OXPHOS in order to meet this additional demand for ATP during DN. OXPHOS is the process by which a membrane potential ($\Delta\Psi_m$) is generated across the inner mitochondrial membrane (IMM) via the ETC. The ETC is composed of a series of protein complexes (complex I–IV). This $\Delta\Psi_m$ drives the transfer of H^+ across the IMM into the inter-membrane space (IMS). The passive diffusion of H^+ via ATP synthase back across the IMM into the matrix results in a proton motive force that drives ATP production. The ETC receives electrons from NADH, and FADH_2 , which are produced via the citric acid cycle. In DN, where there is an abundance of substrate in the form of glucose, the citric acid cycle is readily fuelled to produce NADH and FADH_2 . However, during the transfer of electrons, leakage can occur from complex I and complex III. These leaked electrons react with oxygen to create ROS in the form of superoxide (extensively reviewed by Murphy, 2009). Hence, over a sustained period of time, the accumulative effects of excessive ROS production may overwhelm the mitochondria's antioxidant defence, resulting in oxidative stress. The essence of this idea is born from Denham Harman's free radical theory of ageing, whereby uncontrolled mitochondrial reactive oxygen species (mROS) production resulted in damage to mitochondria, particularly mitochondrial DNA (mtDNA) (Harman, 1972). The mtDNA codes for subunits of the ETC, thus damage caused by oxidative stress can result in impaired formation of complexes leading to inefficient OXPHOS, further superoxide production and decreased ATP production. Hence, over time, excessive amounts of ROS can lead to a vicious cycle, resulting in mitochondrial dysfunction (Figure 1B).

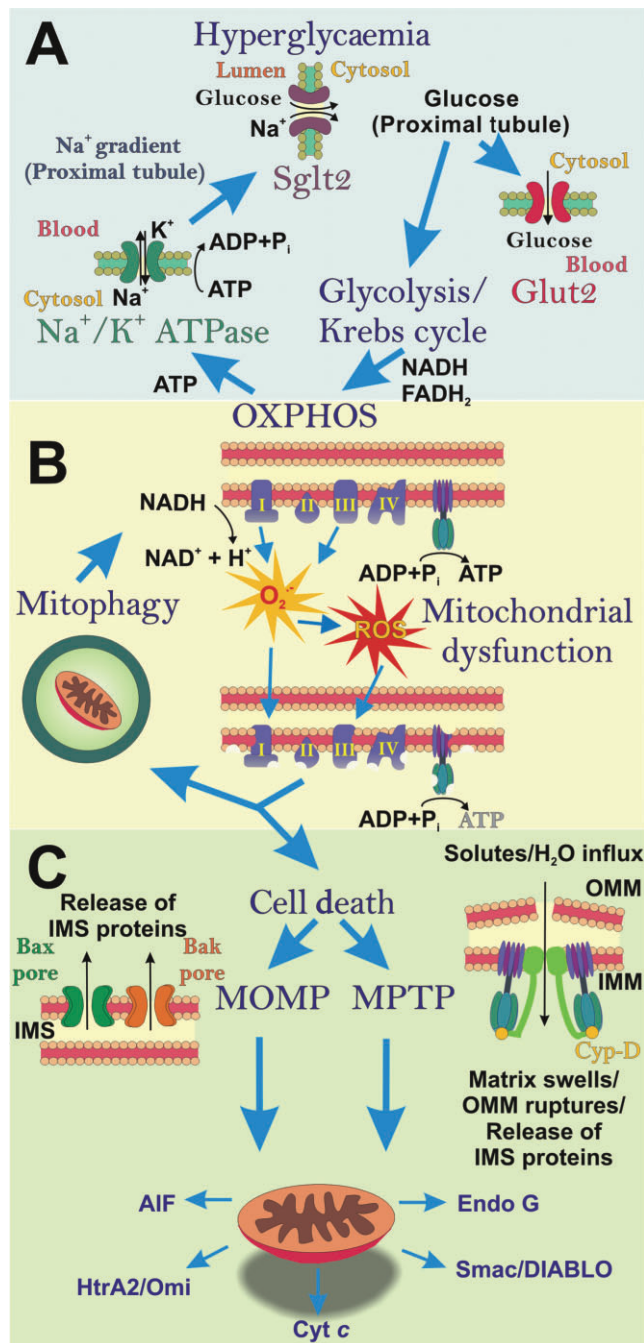


Figure 1

The 'vicious cycle' of diabetic nephropathy (DN): impact of hyperglycaemia on mitochondrial function. (A) In the diabetic milieu, excess blood glucose, filtered by the kidneys has to be reabsorbed, predominantly by the S1 segment of the proximal tubule. Glucose is transported through the proximal tubule epithelial cell (PTEC) via Sglt2 (on the apical/luminal surface) and GLUT2 (on the basolateral surface). Within the PTEC, this increased pool of glucose is metabolized and ATP is produced by oxidative phosphorylation (OXPHOS). The resultant ATP is utilized to fuel the Na⁺/K⁺-ATPase pump, which drives the Na⁺ gradient across the proximal tubule, required for co-transport of glucose via Sglt2. (B) During hyperglycaemia, superoxide (O₂⁻) is generated from the electron transport chain (ETC) and can be subsequently converted into other reactive oxygen species (ROS). Excess ROS causes damage to mitochondria and the ETC, resulting in impaired ATP production. These dysfunctional mitochondria can be eliminated by mitophagy. However, when the number of damaged mitochondria overwhelms the mitophagy process, dysfunctional mitochondria can initiate cell death. (C) Apoptosis or programmed necrosis ensues via mitochondria outer membrane permeabilization (MOMP) or mitochondrial permeability transition pore (MPTP). These mechanisms result in the release of inter-membrane space (IMS) proteins from mitochondria into the cytosol, which promote cell death.

2005). This decline in OXPHOS results in a reduced ATP pool. Indeed, mitochondrial ATP depletion has been shown in the renal cortex in a diabetic setting (Tan *et al.*, 2010). ATP depletion in tubular epithelial cells can lead to cytoskeletal changes as actin polymerization is ATP-dependent (Atkinson *et al.*, 2004). Disruption of the cytoskeleton results in the degradation of the brush border, loss of cell to cell contact, disruption of barrier function and cell detachment (Sharfuddin and Molitoris, 2011).

Increased formation of ROS has been widely observed in the diabetic kidney (Raza *et al.*, 2004; Moreira *et al.*, 2006; Coughlan *et al.*, 2007; 2009; Fujita *et al.*, 2009; Galloway *et al.*, 2012; Sourris *et al.*, 2012; Wang *et al.*, 2012), and, in tandem with an inherent manganese superoxide dismutase (MnSOD or SOD2) functional decline (Coughlan *et al.*, 2007; de Cavanagh *et al.*, 2008) would serve to induce oxidative stress. Moreover, an increase in oxygen consumption is generally seen (Korner *et al.*, 1994; Katyare and Satav, 2005; Palm *et al.*, 2008) and is thought to be a manifestation of mitochondrial uncoupling (Hansell *et al.*, 2013). In contrast, two studies found no change in mitochondrial respiration in the renal cortices of Ins2-Akita mice, a genetic model of type 1 diabetes (Bugger *et al.*, 2009; Chacko *et al.*, 2010). Although these studies demonstrate somewhat variable results, taken together, they indicate an impairment in mitochondrial bioenergetics in the kidney in the diabetic setting. Since each study differed in methodology, that is, differences in substrate utilization, rodent model, age and diabetes duration, it is not surprising that inconsistencies are apparent between data sets. The overall paradigm that can be drawn from these studies is that the metabolic and bioenergetic changes in experimental diabetes may occur before the onset of structural changes, lending support for a causal role for disturbed mitochondrial homeostasis in the development of DN.

Less is known about mitochondrial function in human DN. This is due to the lack of availability of fresh renal biopsy

Studies from animal models indicate that disturbances in mitochondrial homeostasis are central to the pathogenesis of DN. Aberrations in mitochondrial function within the kidney in the context of experimental diabetes have long been observed. Lesions have been seen at the level of the ETC, with a decline in the activity of complex I (Coughlan *et al.*, 2009) and complex III (Rosca *et al.*, 2005), observed within the renal cortex in rats with streptozotocin (STZ)-induced diabetes. Current dogma is that hyperglycaemia provides excess substrate to the respiratory chain, which, in the face of impaired ETC subunits, leaks electrons to form superoxide (Brownlee,

material, which is essential for tests of bioenergetics. However, indirect measures of mitochondrial homeostasis have been determined in patients with DN. Recently, Sharma *et al.* performed metabolomics on urine from patients with type 1 or type 2 diabetes and found that metabolites from the citric acid cycle, pyrimidine metabolism, amino acid, propionate, fatty acid and oxalate metabolism, were significantly reduced in DN (Sharma *et al.*, 2013). Because the majority of these metabolites are produced within the mitochondria, the authors suggested that a generalized decrease in several aspects of mitochondrial function is present in patients with DN. Validation of these pathways led to the discovery that protein levels of complex IV of the ETC were decreased in renal biopsies from patients with DN, and mtDNA content of urine exosomes was likewise decreased. Peroxisome proliferator-activated receptor γ co-activator 1 α (PGC1 α) mRNA was also down-regulated within microdissected cortical tubulointerstitial samples from patients with DN compared with non-diabetic individuals, suggesting that mitochondrial biogenesis may also be impaired. Other mitochondrial proteins have been shown to be altered in the kidney in patients with DN. In a gene expression database, renal biopsies of human DN demonstrated higher expression of genes encoding key mitochondrial proteins, including cytochrome *c* (cyt *c*) and MnSOD, compared with control biopsies (Hickey *et al.*, 2011). These studies, however, are unable to establish a causal relationship for mitochondrial dysfunction in the pathogenesis of DN.

Mitochondria and renal fibrosis

Tubulointerstitial fibrosis is the terminal pathway leading to ESRD in the majority of all CKDs (Liu, 2006). Fibrosis, the consequence of excessive deposition of ECM, is considered a failed wound-healing process (for a comprehensive review, see Liu, 2011). Fibrosis of the kidney results in glomerulosclerosis, tubulointerstitial fibrosis, tubular atrophy and dilatation, and rarefaction of the glomerular and peritubular capillaries (Boor *et al.*, 2010). This process is highly complex, as most cell types that reside within the kidney, in addition to infiltrating cells such as fibrocytes, macrophages and lymphocytes, participate in fibrogenesis. The main cellular events that occur in the pathogenesis of renal fibrosis include infiltration of inflammatory cells, fibroblast activation and expansion from various sources, production and deposition of ECM, tubular atrophy and microvascular rarefaction (Liu, 2011). All of these events lead to a loss of renal function. So which mechanisms could link mitochondrial dysfunction to fibrosis? During the initial stages of fibrosis, following injury, inflammatory cells infiltrate and generate fibrogenic cytokines and growth factors (Duffield, 2010). It is conceivable that ROS generated from mitochondria in the diabetic milieu initiates the inflammatory response. Indeed, mROS has been shown to activate the NLRP3 inflammasome (Zhou *et al.*, 2011), which is also induced in DN (Fang *et al.*, 2013a). There also is an extensive body of evidence that mROS trigger pro-inflammatory cytokine production in an inflammasome-independent manner (Naik and Dixit, 2011).

During renal fibrosis, profibrotic cytokines primary fibroblasts and other cells including tubular epithelial cells undergo phenotypic activation or transition and generate and secrete ECM. It is thought that fibroblasts, pericytes and perivascular cells acquire a myofibroblast phenotype, which may also lead to cytoskeletal rearrangements, altered cell polarity and synthesis of profibrotic and pro-inflammatory mediators (Boor *et al.*, 2010). TGF- β is a key mediator of fibrosis and is up-regulated in DN (Ziyadeh, 2004). It induces ECM accumulation and proliferation of myoblasts and fibroblasts. The link between TGF- β and mitochondrial bioenergetics has been recently reviewed (Casalena *et al.*, 2012). An increase in TGF- β activity is associated with mitochondrial dysfunction and increased mROS synthesis with apoptosis of podocytes and tubular epithelial cells, driving the progression of glomerulosclerosis and nephron loss (Casalena *et al.*, 2012). A growing body of work involving the mitochondrial-associated protein induced-by-high-glucose-1 (IHG-1) has provided a critical molecular link between mitochondria and renal fibrosis in DN (Murphy *et al.*, 2013). IHG-1 was identified in a screen for genes differentially expressed in renal cells exposed to high glucose (Murphy *et al.*, 1999) and was subsequently found to be increased tenfold in tubule-enriched microdissected renal biopsies from patients with DN (Murphy *et al.*, 2008). IHG-1 expression co-localized with activated Smad3, a downstream mediator of TGF- β 1-induced fibrosis. Further investigation determined that IHG-1 amplifies TGF- β 1 action in renal tubular HK-2 cells, leading to typical fibrotic responses such as increased expression of connective tissue growth factor and fibronectin. IHG-1 was subsequently found to be localized to the mitochondria and to modulate mitochondrial mass by stabilization of PGC1 α (Hickey *et al.*, 2011). Mitochondrial localization of IHG-1 was shown to be pivotal for the amplification of TGF- β 1 signalling (Corcoran *et al.*, 2013). As IHG-1 regulates mitochondrial biogenesis, it is likely to play a role in renal cell survival during oxidative stress. In pathological conditions, this would result in dedifferentiation of the renal tubule epithelial cell and fibrosis (Murphy *et al.*, 2013).

ROS also activate a range of other pathways implicated in the pathogenesis in renal fibrosis, including the NF κ B-monocyte chemoattractant protein-1 pathway, which promotes monocyte recruitment and profibrotic processes; the plasminogen activator inhibitor pathway, leading to ECM accumulation via suppression of plasmin activity; and finally, the TGF- β 1-induced myofibroblast transformation of renal PTECs (epithelial-mesenchymal transition) contributing to tubulointerstitial fibrosis (Ha and Lee, 2005).

It is widely appreciated that activation of glomerular mesangial cells by angiotensin II, a vasoactive peptide and growth factor, leads to ECM accumulation. A recent study demonstrated a link between angiotensin II and the mitochondria, whereby angiotensin II stimulated NADPH oxidase 4 (NOX4) up-regulation within the mitochondria, with mROS generation, loss of NO availability and fibronectin accumulation (Lee *et al.*, 2013). There is an increasing body of evidence for crosstalk between different enzymatic sources of oxidative stress. Mitochondrial ROS has been shown to trigger NOX activation in phagocytes and cardiovascular tissue, leading to endothelial dysfunction (Kroller-Schon *et al.*, 2013).

Mitochondrial dynamics and DN

It is important to note that other aspects of mitochondrial biology can modulate mitochondrial functional capacity, such as mitochondrial morphology or dynamics. Altered mitochondrial morphology was first demonstrated in the proximal tubule cells of patients with type 2 diabetes and albuminuria more than 20 years ago (Takebayashi and Kaneda, 1991). Renal biopsy specimens showed enlarged mitochondria with cellular hypertrophy and thickening of the tubular basement membrane. The same research group then showed that enlarged mitochondria in proximal tubule cells correlated with microalbuminuria in early diabetes in a rat model of STZ-induced diabetes (Kaneda *et al.*, 1992). This has been recently confirmed, both in an STZ-mouse model (Sun *et al.*, 2008; Galloway *et al.*, 2012) and in a mouse model of type 2 diabetes, the db/db mouse (Persson *et al.*, 2012). In the latter study, although an increase in mitochondrial fragmentation was found, only two mice per group were studied. In another study, evidence was obtained of mitochondrial fragmentation in the podocytes of mice with STZ diabetes, in parallel with enhanced mitochondrial superoxide production and up-regulation of glomerular dynamin-related protein 1 (Drp-1) (Wang *et al.*, 2012), a GTPase of the dynamin superfamily that plays an essential role in mitochondrial fission. Taken together, these studies suggest that perturbed mitochondrial dynamics may be important in the pathogenesis of DN. The role of mitochondrial fragmentation in the pathogenesis of renal pathophysiology is a newly emerging area (recently reviewed by Zhan *et al.*, 2013); however, the molecular events that govern mitochondrial dynamics in DN are not yet known, and the mechanisms whereby alterations in dynamics lead to renal fibrosis have not been elucidated. It is clear that further studies in this area are warranted.

Mitochondrial function and cell death

Unabated mitochondrial dysfunction can ultimately result in the activation of the intrinsic cell death pathways and cellular demise (Davis and Williams, 2012; Smith *et al.*, 2012b). In addition to being considered the cellular powerhouse, mitochondria are also known to be the judge and executioner during programmed cell death (Tait and Green, 2010; Kubli and Gustafsson, 2012). Programmed cell death occurs in several forms, including apoptosis, autophagic cell death (ACD) and programmed necrosis [caspase-independent cell death that is highly regulated, yet with features similar to accidental necrosis (Baines, 2010)], which encompasses necroptosis (RIP1-dependent) (Christofferson and Yuan, 2010) and parthanatos (PARP-1-dependent) (Andrabi *et al.*, 2008). Despite these cell death pathways being distinct, Bcl-2 family members are implicated in each, directly or indirectly regulating mitochondrial involvement (Youle and Strasser, 2008; Martinou and Youle, 2011). Bcl-2 family members, such as Bnip3, Nix, Bax, Bak, Bmf and Bcl-X_L, have been implicated in programmed necrosis (Vande Velde *et al.*, 2000; Kubasiak *et al.*, 2002; Moubarak *et al.*, 2007; Hitomi *et al.*, 2008; Diwan *et al.*, 2009; Chen *et al.*, 2010; Messner *et al.*, 2012; Whelan *et al.*, 2012; Michels *et al.*, 2013). As will be discussed

later, a number of autophagy proteins have been shown to interact with Bcl-2 family members.

In the case of apoptosis and programmed necrosis, the redistribution of IMS cell death proteins such as cyt *c*, Smac/DIABLO and Htra2/Omi, which are involved in caspase-dependent cell death and caspase-independent signalling apoptosis-inducing factor (AIF) and endonuclease G are central (Kubli and Gustafsson, 2012) (Figure 1C). However, these IMS cell death signalling proteins depend on two distinct mechanisms for redistribution. Apoptosis involves the pro-apoptotic Bcl-2 family members Bax and Bak to form a pore on the outer mitochondrial membrane (MOMP), resulting in transient hyperpolarization followed by dissipation of $\Delta\Psi_m$, the release of proteins from the IMS to the cytosol (Tait and Green, 2010) (Figure 1C).

In contrast to mitochondrial membrane permeabilization during apoptosis, programmed necrosis appears to involve the initiation of mitochondrial permeability transition pore (MPTP) (Baines, 2010). This pore causes dissipation of $\Delta\Psi_m$, allows solutes (less than 1.5 kDa in size) to enter the mitochondria resulting in mitochondrial matrix swelling and subsequent rupture of the outer mitochondrial membrane (OMM) releasing the contents of the IMS (Zorov *et al.*, 2009). However, unlike MOMP, much conjecture remains as to the composition of the MPTP. It was initially thought that the MPTP was composed of the voltage-dependent anion channel 1 (VDAC1), adenine nucleotide translocator (ANT) and cyclophilin D (Cyp-D), spanning the OMM and IMM (Armstrong, 2006; Zorov *et al.*, 2009). The subsequent demonstration that VDAC1 and ANT are not needed for mitochondrial permeability transition (MPT) to occur has cast doubt over this classical model (Kokoszka *et al.*, 2004; Baines *et al.*, 2007). Nonetheless, Cyp-D was identified as a critical component of the MPTP, with Cyp-D-deficient cells unable to undergo MPT (Baines *et al.*, 2005; Nakagawa *et al.*, 2005). More recently, dimers of ATP synthase have been shown to form the MPTP within the IMM, with Cyp-D binding to the lateral stalk of ATP synthase (Giorgio *et al.*, 2013) (Figure 1C). The implications of this discovery are further detailed in Bernardi (2013), although it is evident that this already remarkable enzyme is at the centre of the crossroads between biogenesis and cell death, which also affects mitophagy.

Non-selective autophagy signalling: indiscriminate removal of mitochondria

Classical autophagy, as it was first described by De Duve, is a non-selective process whereby cytosolic contents (long-lived proteins and organelles) are sequestered into autophagosomes for transport to lysosomes for bulk degradation (De Duve and Wattiaux, 1966). Although selective autophagy (which encompasses mitophagy) may appear to be a more refined and efficient means of removing dysfunctional mitochondria, much remains unknown as to how these two autophagy processes interact. In order to develop effective therapeutics to modulate the accumulation of dysfunctional mitochondria, both processes must be given due consideration. Non-selective autophagy activity can be modulated by growth factors, such as negative regulation via

insulin and insulin-like growth factor-1 (Troncoso *et al.*, 2012; Naito *et al.*, 2013; Paula-Gomes *et al.*, 2013; Renna *et al.*, 2013) and positively by TGF- β (Ding *et al.*, 2010b; Koesters *et al.*, 2010; Xu *et al.*, 2012), cellular stress (e.g. oxidative stress, hypoxia, advanced glycation end-products) (Miyata and de Strihou, 2010; Hu *et al.*, 2012; Xu *et al.*, 2013) and changes in nutrient levels (e.g. glucose, AMP/ATP ratio) (Moruno *et al.*, 2012; Lempiainen *et al.*, 2013; Vallon *et al.*, 2013). It is likely that these same stimuli, many of which have been linked to the development of functional or structural defects of DN (Forbes and Cooper, 2013), play critical roles in modulating selective autophagy activity in the DN milieu (Figure 2).

In the face of cellular starvation, stress or growth factor withdrawal, cells undergo non-selective autophagy regulated by the mechanistic target of rapamycin (mTOR) signalling pathway to recycle cellular components and maintain energy levels (Foster and Fingar, 2010). Inhibition of mTOR stimulates gross autophagy, which indiscriminately encapsulates damaged and healthy mitochondria alike, along with other organelles and long-lived proteins for bulk degradation. This pathway is a relatively rudimentary means of removing damaged mitochondria. Acute treatments with rapamycin inhibit mechanistic target of rapamycin complex 1 (mTORC1), making it a widely used inducer of autophagy activity in experimental studies (Klionsky *et al.*, 2012). There have been several seminal studies demonstrating that attenuating mTORC1 signalling can cause longevity in eukaryotic organisms such as *Caenorhabditis elegans* (Vellai *et al.*, 2003), *Drosophila* (Kapahi *et al.*, 2004) and mice (Harrison *et al.*, 2009).

Mitophagy: selective removal of dysfunctional mitochondria

Selective autophagy is the process by which damaged or surplus proteins and organelles are specifically targeted for degradation, as eloquently reviewed recently (Feng *et al.*, 2013; Shaid *et al.*, 2013). In the last decade, the discovery of selective autophagy has seen the emergence of many subtypes of autophagy. Such terms as mitophagy, reticulophagy and ribophagy have been coined for the select removal of mitochondria, ER and ribosomes respectively (Lemasters, 2005; Cebollero *et al.*, 2012; Jin and Youle, 2012). Although each could potentially play a role in DN, attention will only be given to mitophagy here based on the evidence provided that the accumulation of damaged mitochondria contribute to its pathology. Mitophagy is distinguished by its regulation through the ubiquitin proteasome system (UPS), although the emergence of UPS-independent adaptor proteins suggests that alternative pathways may exist, or that this process has cell-type specific variances. Cellular recognition of dysfunctional mitochondria is determined by loss of $\Delta\Psi_m$. Changes in mitochondrial morphology and microtubule transport of damaged mitochondria have secondary roles in determining whether mitophagy is engaged. This mitophagy activity may, in turn, be regulated by ATP and mROS production, which may determine the rate at which mitochondria are turned over. These aspects of mitophagy regulation will be covered in detail in the following sections.

Recognition of damaged mitochondria via loss of $\Delta\Psi_m$

The key determinant that a mitochondrion should be targeted for degradation is the cessation of OXPHOS. Dysfunctional mitochondria that do not have an $\Delta\Psi_m$ are tagged for mitophagy clearance via PTEN-induced putative kinase 1 (PINK1) on the organelle's outer membrane (Matsuda *et al.*, 2010; Narendra *et al.*, 2010b; Vives-Bauza *et al.*, 2010). PINK1, which is synthesized in the cytosol, is imported into mitochondria via the translocase of the outer mitochondrial membrane (TOM) complex into the mitochondrial IMS. In functional mitochondria (with $\Delta\Psi_m$), the mitochondrial targeting sequence (MTS) of PINK1 guides the protein through the IMS into the translocase of the inner membrane (TIM) complex where it spans the inner membrane (Jin *et al.*, 2010). The MTS of PINK1 is cleaved by MPP (mitochondrial processing peptidase), while the protein itself undergoes processing via presenilin-associated rhomboid-like protease (PARL) located within the IMM (Jin *et al.*, 2010; Meissner *et al.*, 2011; Greene *et al.*, 2012). This processed form of PINK1 is rapidly degraded by a further mitochondrial protease. In the case of dysfunctional mitochondria that do not have $\Delta\Psi_m$, PINK1 is unable to pass through the TIM complex in the IMM, leaving it anchored via the TOM complex on the OMM unprocessed (Lazarou *et al.*, 2012). Here, PINK1 is recognized by Parkin, an E3 ubiquitin ligase, which translocates to the mitochondria from the cytosol and is phosphorylated by PINK1 at Ser⁶⁵ to become active and initiate mitophagy (Narendra *et al.*, 2008; 2010b; Matsuda *et al.*, 2010; Vives-Bauza *et al.*, 2010; Kondapalli *et al.*, 2012; Iguchi *et al.*, 2013). However, whereas the generally accepted view is that PINK1 phosphorylates Parkin at Ser⁶⁵, this has been shown to be non-essential in some circumstances (Shiba-Fukushima *et al.*, 2012; Sarraf *et al.*, 2013). Upon binding with PINK1, Parkin mediates the ubiquitination of OMM proteins such as VDAC1, the TOM complex, the pro-apoptotic protein Bak and proteins involved in mitochondrial dynamics and transport such as mitofusins 1/2 (Mfn1/2) and mitochondria Rho GTPase (Miro) respectively. The purpose of ubiquitinating these proteins and the possible degradation of these proteins via the UPS will be discussed in the subsequent sections below.

Encapsulation of mitochondria within autophagosomes is dependent on adaptor proteins

One defining characteristic of selective autophagy that differentiates it from non-selective autophagy is the dependence on adaptor (or receptor) proteins to encapsulate its target molecule or organelle. As a form of selective autophagy, mitophagy can engage adaptor proteins such as p62/SQSTM1 (sequestosome 1) (Geisler *et al.*, 2010), Nix (Bnip3L) (Novak *et al.*, 2010), Bnip3 (Hanna *et al.*, 2012), FUN14 domain containing 1 (FUNDC1) (L. Liu *et al.*, 2012) and histone deacetylase 6 (HDAC6) (Lee *et al.*, 2010), which bind to the OMM and aid the docking of the isolation membrane of the pre-autophagosome with damaged mitochondria. Each of the aforementioned adaptor proteins has a unique composition that encompasses binding sites that interact with either light chain 3 (LC3) via an LC3 interacting region (LIR) or ubiquitin via an ubiquitin-binding domain (UBD) during mitophagy.

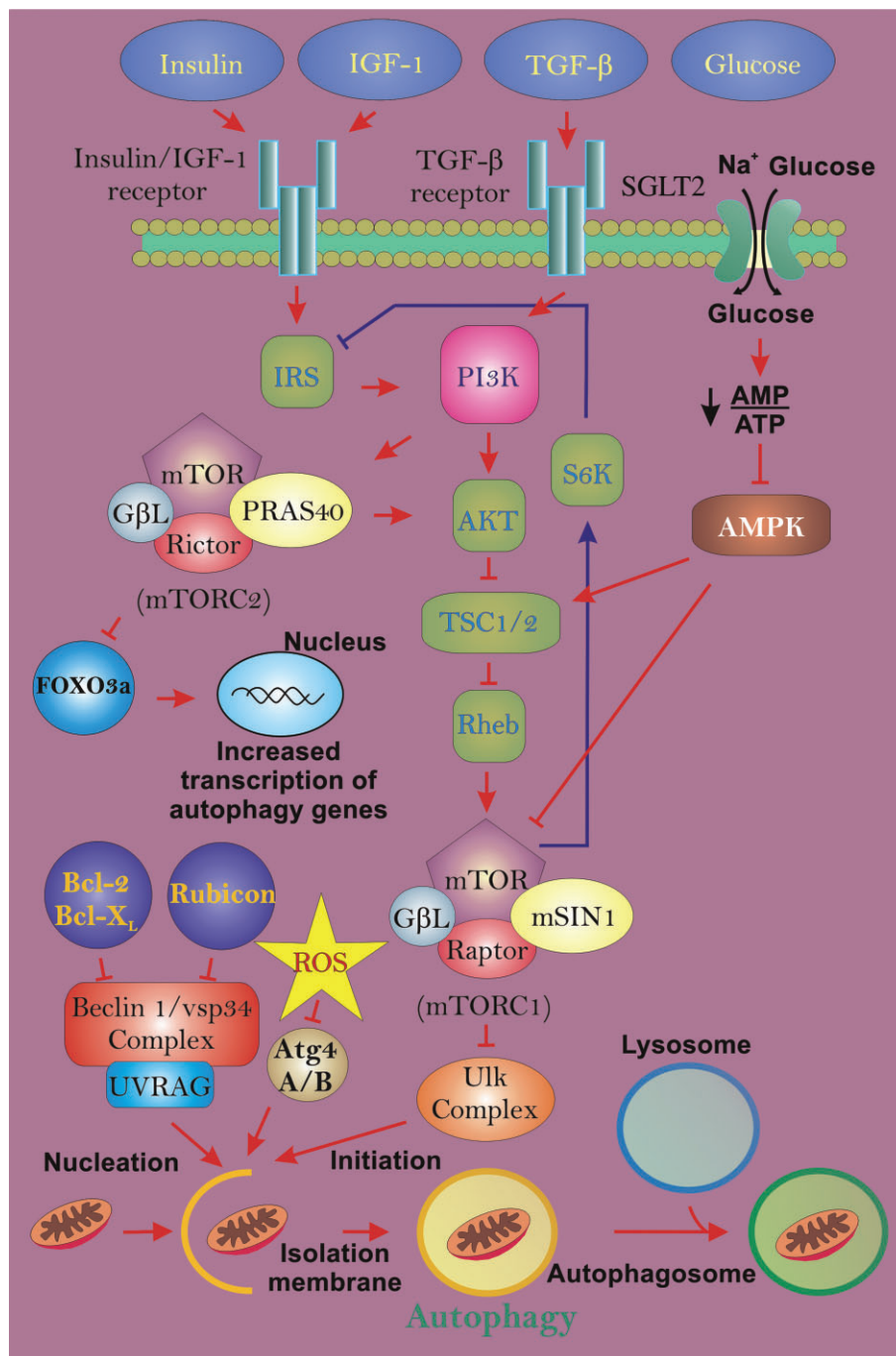


Figure 2

Non-selective autophagy regulation during diabetic nephropathy (DN). During DN, autophagic turnover of mitochondria can be influenced by changes in several contributing factors, including insulin, IGF-1 (insulin-like growth factor-1), TGF- β , glucose and reactive oxygen species (ROS). These influences can act on the mechanistic target of rapamycin (mTOR) signalling pathway, which negatively regulates autophagy activity. Insulin, IGF-1 and TGF- β can modulate the mTOR pathway via the PI3K pathway. Increases in insulin or IGF-1 will suppress autophagy activity, while an increase in TGF- β will stimulate autophagy activity. Increases in glucose, with hyperglycaemia, can result in suppression of autophagy through activation of AMPK, which can activate the mTOR pathway. Finally, increases in ROS production can stimulate autophagy activation via inhibition of Atg4A/B or by directly acting on other components of the mTOR pathway, impairing activation.

The adaptor proteins Bnip3, Nix and FUNDC1 possess an LIR, which allows these proteins to dock LC3 to the OMM (Novak *et al.*, 2010; Hanna *et al.*, 2012; L. Liu *et al.*, 2012). The mechanism that allows these adaptor proteins to recruit LC3 to the OMM remains unclear. Yet, it is evident that FUNDC1, Bnip3 and Nix have different roles in mitophagy as shown by their differential binding affinities to different orthologues of LC3. FUNDC1 and BNIP3 have a high affinity for LC3B (L. Liu *et al.*, 2012; Zhu *et al.*, 2013), whereas Nix has a weak affinity for LC3B, but a high affinity for LC3A and GABARAP-L1 (Novak *et al.*, 2010). Under hypoxic conditions, these LIR-only adaptor proteins have been shown to be involved in mitophagy, with Bnip3 and Nix expression up-regulated, whereas FUNDC1 is down-regulated, again suggesting functional differences between these proteins (Bellot *et al.*, 2009; L. Liu *et al.*, 2012). Whereas hypoxia-inducible factor 1 α (HIF-1 α) has been shown to mediate Bnip3 and Nix-dependent mitophagy (Bellot *et al.*, 2009), it remains to be determined whether FUNDC1 is similarly regulated. The implications of these discoveries are pertinent to DN, where the onset of hypoxia has been demonstrated to occur in early stage renal failure (Palm and Nordquist, 2011). Moreover, Bnip3 has recently been shown experimentally to regulate autophagy and mitophagy in proximal tubular cells during acute kidney injury (AKI) (Ishihara *et al.*, 2013).

Recognition of dysfunctional mitochondria by the PINK1/Parkin pathway appears to be dependent on the involvement of Bnip3 and Nix, despite their lack of a UBD (Ding *et al.*, 2010a; Lee *et al.*, 2011). Both Bnip3 and Nix have been shown to promote the translocation of Parkin to mitochondria. Interestingly, Bnip3 and Nix both have the ability to induce mitochondrial depolarization, which can be a trigger for mitophagy as discussed earlier (Sandoval *et al.*, 2008; Rikka *et al.*, 2011). However, given the involvement of Bnip3 and Nix in cell death signalling, FUNDC1 might be the more dependable adaptor protein in which to develop a therapeutic strategy to stimulate mitophagy.

Unlike Bnip3, Nix and FUNDC1 that act independently of ubiquitin, HDAC6 and p62 can directly attach to polyubiquitin chains via their UBD domains. The recruitment of HDAC6 and p62 during mitophagy is therefore dependent on ubiquitination of OMM proteins mediated by Parkin (Geisler *et al.*, 2010; Lee *et al.*, 2010). HDAC6 only possess a UBD in the form of a ubiquitin-binding zinc finger (Seigneurin-Berny *et al.*, 2001; Hook *et al.*, 2002), whereas p62 is distinct in that it comprises a UBD as well as an LIR, allowing it to interact with both LC3 and ubiquitin respectively (Bjorkoy *et al.*, 2005). Ubiquitin can attach to its substrate as a single ubiquitin monomer (monoubiquitination) or form chains of sequentially conjugated ubiquitin (polyubiquitination). Each ubiquitin monomer has seven lysine (K) residues that can act as a point of polyubiquitination. Polyubiquitin chains are therefore identified based on which lysine of the ubiquitin monomer it is attached to. Ligation of OMM substrates by Parkin can result in either K27-, K48- or K63-linked polyubiquitin chains (Geisler *et al.*, 2010; Narendra *et al.*, 2010a; Rana *et al.*, 2013). K63-linked polyubiquitin chains are considered 'non-classical', as substrates with these labels are predominantly targeted for selective autophagy rather than degradation via the UPS (Olzmann *et al.*, 2007; Tan *et al.*, 2008; Geisler *et al.*, 2010; Narendra *et al.*, 2010a). The adaptors

p62 and HDAC6 can recognize and attach to K63-polyubiquitinated proteins for transport via dynein motors along microtubules to the perinuclear or juxtannuclear region to form aggresomes, which are clusters of misfolded proteins (Kawaguchi *et al.*, 2003; Seibenhener *et al.*, 2004). The LIR on p62 binds with LC3 to encapsulate and degrade aggresomes via autophagy.

Mitochondria are also capable of aggregating via p62 and HDAC6 in a manner similar to misfolded proteins when ubiquitinated (Lee *et al.*, 2010; Narendra *et al.*, 2010a; Okatsu *et al.*, 2010). However, whereas several groups have reported that transport and aggregation of damaged mitochondria by p62 is critical for mitophagy (Geisler *et al.*, 2010; Lee *et al.*, 2010), p62 has also been reported to be dispensable (Okatsu *et al.*, 2010; Narendra *et al.*, 2010a). HDAC6 transport has so far been shown to be essential for mitophagy (Lee *et al.*, 2010). It is important to note that the role of either adaptor has not been fully investigated in a pathological *in vivo* model, and whereas mitophagy may still occur in the absence of perinuclear clustering, the degradation efficiency may be compromised.

K48-linked polyubiquitin chains can also form on the OMM (Sun *et al.*, 2009). K48-linked ubiquitin chains are considered 'classical' in that proteins tagged with these chains are generally degraded by proteasomes (Shaid *et al.*, 2013). OMM substrates, such as Mfn1 and Mfn2 that are ligated by Parkin have been shown to possess K48-linkages (Chan *et al.*, 2011; Lazarou *et al.*, 2013). Through the use of proteasome inhibitors, the degradation of these proteins was shown to be dependent on the UPS. Of further interest was the demonstration that mitophagy is dependent on proteasomal activity (Chan *et al.*, 2011). Parkin has also been reported to ligate VDAC1 with K27-linked polyubiquitin chains (Geisler *et al.*, 2010), although mitophagy is not dependent on this step (Narendra *et al.*, 2010a). This apparent multi-specificity of Parkin ligation is most likely dependent upon which E2 enzymes are recruited to each substrate (Lim *et al.*, 2006). HDAC6 and p62 have both been reported to influence the activity of the UPS as both are capable of binding to K48-polyubiquitin chains, providing a potential compensatory crosstalk mechanism between the two degradation pathways (Korolchuk *et al.*, 2009; Du and Jiao, 2011; Shaid *et al.*, 2013). This close relationship between the UPS and autophagy is further demonstrated by the regulation of mitophagy by p97/VCP (Tanaka *et al.*, 2010). p97 is a AAA-ATPase that was originally discovered as a chaperone for delivery of substrates to the UPS, but has more recently been determined to have a role in regulating autophagy and lysosomal degradation (Meyer *et al.*, 2012).

Impedance of mitochondrial dynamics and transport facilitates mitophagy

Dysfunctional mitochondria undergo retrograde transport to the perinuclear region where they cluster during mitophagy (Narendra *et al.*, 2010a; Okatsu *et al.*, 2010; Vives-Bauza *et al.*, 2010). The perinuclear region of the cell is the location of the ER, as well as being rich in lysosomes and proteasomes. During mitophagy, encapsulated mitochondria are probably transported along the microtubules via dynein motors to the perinuclear region where degradation via lysosomes can occur. This phenomenon may allow for more efficient

recycling of mitochondria, which can be broken down into basic constituents that can be utilized by the ER. Under severe stress, perinuclear clustering may be advantageous to cope with an influx in mitophagy activity. The population of mitochondria within any cell type are dynamic, constantly being transported along microtubules, while undergoing constant fission and fusion between adjoining mitochondria within the network. To initiate mitophagy, disengagement of mitochondria from the mitochondrial network is required. Overall, regulation of mitochondrial dynamics requires PINK1 and Parkin interaction with the OMM proteins, Miro involved in microtubule transport (Wang *et al.*, 2011), Drp1, Mfn 1 and 2 that regulate mitochondrial dynamics and ultimately the adaptors p62 and HDAC6 for perinuclear transport as discussed earlier.

Miro links with another adaptor protein Milton and kinesin-1 heavy chain (KHC) to form the KHC complex. The KHC complex allows anterograde transport of mitochondria via the kinesin motor (Saotome *et al.*, 2008; Wang and Schwarz, 2009). Miro has also been shown to mediate retrograde transport, suggesting that Miro may interact with the dynein motor as well (Wang and Schwarz, 2009). During mitophagy, PINK1 phosphorylates the Ser¹⁵⁶ of Miro, which is followed by ubiquitination via Parkin, which labels Miro for proteasomal degradation (Wang *et al.*, 2011). The degradation of Miro results in arrest of mitochondrial motility. The purpose of this action during mitophagy remains unclear, although it may help to quarantine damaged mitochondria or aid the encapsulation of the organelle within autophagosomes by rendering the organelle stationary. Given that Miro is located on the OMM, mitochondrial encapsulation and subsequent retrograde transport during mitophagy does not involve Miro.

Dynamic morphological changes in mitochondria are required to maintain a homogenous population of functional mitochondria to ensure continuous OXPHOS. Mitochondrial fusion allows cross-complementation by sharing mtDNA, lipids and proteins between adjoining organelles, which is thought to preserve mitochondrial integrity (Youle and van der Bliek, 2012). Given the relatively high level of superoxide generated during OXPHOS, this dynamic process may occur to minimize the effects of mtDNA mutations, which code for subunits of the ETC, as well as diluting the pool of damaged proteins and lipids. Mitochondrial dynamics are tightly regulated by two distinct groups of proteins. Mitochondrial fission is regulated by the cytosolic Drp1, which is recruited to the OMM where it interacts with Fis1, Mid49, Mid51 and mitochondrial fission factor to form coils that constrict and separate mitochondria in two (Elgass *et al.*, 2013). Conversely, mitochondrial fusion is mediated by dynamin family members Mfn1 and Mfn2, which fuse the OMM and optic atrophy 1 (OPA1), which mediates fusion of the inner membrane (Youle and van der Bliek, 2012). These opposing mitochondrial dynamics mechanisms are regulated in concert by proteolysis and post-translational modifications. As previously discussed, there is increasing evidence that mitochondrial morphology is altered in the PTECs in DN, suggesting that changes in mitochondrial dynamics may be glucose-driven or modulated by reactive glucose-derived molecules. Indeed, glycation of proteins within the kidney is integral to the pathophysiology of DN (Coughlan *et al.*, 2008).

In order for mitophagy to occur, mitochondria must undergo fission or fragmentation into spheroids that can be encapsulated within autophagic vesicles (Twig *et al.*, 2008). The PINK1/Parkin pathway disrupts the mitochondrial network, which is essential to isolate damaged mitochondria for engulfment by autophagosomes (Poole *et al.*, 2008; Y. Yang *et al.*, 2008; Gegg *et al.*, 2010). Mitochondrial ubiquitin E3 ligases such as MULAN and MARCH5 are thought to facilitate the polyubiquitination of fission and fusion proteins during mitochondrial biogenesis (Karbowski *et al.*, 2007; Li *et al.*, 2008; Park *et al.*, 2010). Similarly, as an E3 ligase, Parkin has been shown to ubiquitinate mitofusins for degradation by the UPS to disengage mitochondrial biogenesis (Poole *et al.*, 2008; Gegg *et al.*, 2010; Tanaka *et al.*, 2010; Ziviani *et al.*, 2010; Rakovic *et al.*, 2011). Although, recently MULAN (also known as Mull1) has also been implicated in mitophagy in skeletal muscle (Lokireddy *et al.*, 2012). The targeting of mitofusins to the UPS prevents damaged mitochondria from fusing with adjoining healthy mitochondria. The transport of mitofusins to the UPS for degradation is mediated by p97 (Tanaka *et al.*, 2010). Furthermore, in cardiomyocytes, phosphorylation of Mfn2 by PINK1 is needed for translocation of Parkin to mitochondria and ubiquitination of Mfn2 and other proteins to ensue (Chen and Dorn, 2013). Ablation of Mfn2 in cardiomyocytes prevented translocation of Parkin and subsequent mitophagy to occur in response to mitochondrial depolarisation. Mfn2 can therefore act as a receptor for Parkin when phosphorylated. However, this observation may be cell-type specific given that Parkin translocation has also been demonstrated in cells lacking Mfn1 and Mfn2 (Narendra *et al.*, 2008; Chan *et al.*, 2011). Furthermore, whereas mitofusins may have an essential role in the occurrence of mitophagy, proteasomal degradation of mitofusins alone does not instigate mitochondrial fission.

The regulation of mitochondrial fragmentation during mitophagy involves the recruitment of Drp1. PINK1 and Parkin have been shown to mediate the recruitment of Drp1 to mitochondria. Loss of PINK1 or Parkin function has been shown to cause an increase in Drp1-dependent mitochondrial fragmentation, which can be reversed by the overexpression of Mfn2, Opa1, a dominant negative of Drp1 or by use of the Drp1 inhibitor mdivi-1 (Lutz *et al.*, 2009; Sandebring *et al.*, 2009; Cui *et al.*, 2010). Similarly, cell culture studies have also revealed that the adaptor protein Bnip3 can induce translocation of Drp1 to mitochondria during mitophagy (Lee *et al.*, 2011). Moreover, Bnip3 has also been reported to disrupt Opa1, leading to mitochondrial fragmentation during apoptosis (Landes *et al.*, 2010). However, it remains to be investigated whether the Bnip3–Opa1 interaction is also involved in mitochondrial fragmentation during mitophagy. Overexpression of Fis1 can also stimulate mitophagy (Gomes and Scorrano, 2008). However, this outcome was shown to be dependent on the ability of Fis1 to cause mitochondrial dysfunction rather than its role in fission. In contrast, Fis1 silencing has been shown to reduce mitophagy activity (Twig *et al.*, 2008). Lastly, the mitochondrial protein p32 has been shown to effect changes in mitochondrial morphology via Parkin, by way of fragmentation when suppressed (Li *et al.*, 2011). Whereas the mechanisms that mediate Parkin translocation to mitochondria remain to be fully defined, the action of p32

may warrant further attention. Taken together, these findings demonstrate that while mitochondrial dysfunction can trigger mitophagy activation, this process is dependent on the regulation of mitochondrial dynamics via the PINK1/Parkin pathway to ensure damaged mitochondria are cleared from the cell.

Regulation of mitophagy activity by ROS

Once mitophagy is activated, this process must be modulated to ensure that the clearance rate is sufficient to prevent the accumulation of damaged mitochondria. Autophagy activity is ROS-dependent and is tightly regulated by intracellular redox signalling (Scherz-Shouval and Elazar, 2011). OXPHOS can be a major source of ROS and, as such, under homeostatic conditions, this is managed by an array of antioxidants. Moreover, mitochondria have their own antioxidant defence network that resides within these organelles to minimize the escape of ROS into the cytosol (Crane *et al.*, 1957; Weisiger and Fridovich, 1973; Ursini *et al.*, 1982). As discussed earlier, in DN, an influx of ROS caused by high glucose has been postulated to contribute to oxidative stress, which contributes to the development of the disease (Forbes *et al.*, 2008). It can be further postulated that this excess ROS may stimulate mitophagy activity in the kidney and regulate the turnover of damaged mitochondria (Chen *et al.*, 2007; Scherz-Shouval *et al.*, 2007; Bensaad *et al.*, 2009). This section will focus on the mitochondria's antioxidant defence and how it may influence redox signalling and the regulation of mitophagy activity.

Transcriptional regulation of autophagy activity can be stimulated by oxidative stress, as well as by mROS produced in response to hypoxia. An influx of ROS or oxidative stress can result in activation of transcription factors HIF-1 α , Forkhead box O3a (FOXO3a) or nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which each, in turn, induce the transcription of autophagy genes (Scherz-Shouval and Elazar, 2011). As discussed earlier, HIF-1 α and FOXO3a regulate the transcription of Bnip3 and Nix, which are both involved in mitophagy. Nrf2 induces transcription of p62 (Jain *et al.*, 2010). Another mechanism that can regulate autophagy in response to ROS is the p53-target gene TIGAR (TP53-induced glycolysis and apoptosis regulator), which, in this capacity, acts independently of p53 and mTOR (Bensaad *et al.*, 2009). TIGAR promotes activation of the pentose phosphate pathway, which produces NADPH. NADPH is required to reduce oxidized glutathione, which is required to lower ROS levels, which, in turn, decreases autophagy activity. Of potential relevance to mitophagy regulation is the recent discovery that under hypoxic conditions, TIGAR can translocate to the mitochondria in an HIF-1 α -dependent manner, where it forms a complex with mitochondrial HK2 (hexokinase 2), which limits mROS levels (Cheung *et al.*, 2012).

The cytosolic protein DJ-1 has been implicated in cellular signalling in response to oxidative stress and linked to mitophagy. DJ-1 has a conserved cysteine residue (Cys¹⁰⁶) that is sensitive to ROS. Oxidation of Cys¹⁰⁶ promotes the translocation of DJ-1 to mitochondria at times of oxidative stress (Canet-Aviles *et al.*, 2004; Zhang *et al.*, 2005; Junn *et al.*, 2009). DJ-1 has been shown to protect against oxidative stress, but this is more likely via ROS suppression through its role in cellular signalling rather than acting as an antioxidant

(Canet-Aviles *et al.*, 2004; Taira *et al.*, 2004; Junn *et al.*, 2009). Loss of DJ-1 results in increased ROS production and MPTP opening in mitochondria of murine embryonic fibroblasts (Gaiame *et al.*, 2012). Furthermore, DJ-1 has been shown to be important for ATP production and mitochondrial function in skeletal muscle and can restore PINK1 loss of function, but not Parkin in *Drosophila* (Hao *et al.*, 2010). Interestingly, DJ-1 has also been shown to associate with Parkin under oxidative stress conditions (Moore *et al.*, 2005; Joselin *et al.*, 2012), and it is suggested to work in parallel with the PINK1/Parkin pathway to regulate mitophagy (Thomas *et al.*, 2011). DJ-1 has also been reported to be involved in proteasomal degradation of Fis1 (Zhang *et al.*, 2012). In the kidney, DJ-1 is expressed in proximal tubule cells where it has been implicated in regulating ROS production via NOX4 during hypertension (Cuevas *et al.*, 2012). Although it is unclear what the exact function of DJ-1 is, it is evident that it may have an important role in regulating mitophagy, particularly under oxidative stress.

Cellular ROS can also promote autophagy via a post-transcriptional mechanism via inactivation of Atg4 (Scherz-Shouval *et al.*, 2007). Atg4 has four paralogues: Atg4A-Atg4D also known as autophagins (Marino *et al.*, 2003). The Atg4 family members are responsible for proteolytic activation and subsequent delipidation (removal from autophagosomes) of Atg8 family members (e.g. LC3 and GABARAP). Atg4A and Atg4B have a redox-sensitive cysteine residue, which can be oxidized by H₂O₂ to inactivate this protease (Scherz-Shouval *et al.*, 2007). It has been hypothesized that when Atg4A or Atg4B are within close proximity of dysfunctional mitochondria producing ROS, these proteases become inactivated, stimulating LC3 lipidation. However, when the autophagosomes translocate to the perinuclear lysosomes, this region must be low in ROS, allowing active Atg4A and Atg4B to delipidate LC3.

The mitochondrial antioxidant network that may regulate mitophagy consists of a diverse range of proteins that are each capable of detoxifying distinct ROS. Electrons that escape the ETC during OXPHOS react with oxygen to produce superoxide (O₂⁻). This O₂⁻ can be detoxified by SOD into hydrogen peroxide (H₂O₂). H₂O₂ can, in turn, be reduced to H₂O via glutathione peroxidase (Gpx). Mitochondria have their own copies of each enzyme, SOD2 (Weisiger and Fridovich, 1973) and mitochondrial Gpx4 (mGpx4) (Ursini *et al.*, 1999). mGpx4 has dual roles; it can reduce hydroperoxides in lipoproteins and is also thought to be responsible for repairing oxidative damage to biomembranes (Ursini *et al.*, 1999). The role of mGpx4 has not been the focus of DN studies to date, as attention has been given to SOD2, largely due to a polymorphism in its targeting sequence.

In a KK/Ta-Akita mouse model of DN, SOD2 activity was found to be unchanged (Fujita *et al.*, 2009). Furthermore, SOD2 activity and expression were also unchanged in a STZ rat model and *in vitro* in rat proximal tubule cells exposed to high glucose (25 mM) despite evidence of mitochondrial dysfunction and elevated mitochondrial superoxide (Munusamy and MacMillan-Crow, 2009). In contrast, studies from our laboratory (Coughlan *et al.*, 2007) and others (de Cavanagh *et al.*, 2008) have found that MnSOD (SOD2) activity is suppressed in the renal cortex in rodent models of DN. Moreover, SOD2 has been implicated in human DN through

a polymorphism, V16A (Val¹⁶Ala) in Danish, Swedish and Finnish type 1 diabetes patients (Mollsten *et al.*, 2007; 2009) and Korean, Chinese and Japanese type 2 diabetes patients (Nomiya *et al.*, 2003; Lee *et al.*, 2006; Liu *et al.*, 2009). An increase in the Val-allele has also been reported in Egyptian type 1 patients (el-Masry *et al.*, 2005). Although this Val-allele increase was statistically insignificant, the cohort in this study was smaller than the other studies reported here. Having valine instead of alanine in the 16th amino acid position of the targeting sequence of SOD2 results in less efficient transport of the antioxidant into the mitochondrial matrix (Shimoda-Matsubayashi *et al.*, 1996; Sutton *et al.*, 2003). Studies on SOD2-kidney specific knockout mice (SOD2^{fl}/Ksp1.3-Cre, knockout in distal tubules, collecting ducts, Loops of Henle, ureteric buds, and developing genitourinary tract) show an influx in autophagy and mitochondrial biogenesis compared with wild-type mice, suggesting that SOD2 deficiencies may stimulate mitophagy (Parajuli and MacMillan-Crow, 2013).

Yet another antioxidant residing in mitochondria is coenzyme Q (CoQ). CoQ has a primary role as an electron carrier between complex I and II and complex II and III in the ETC (Mellors and Tappel, 1966a). Due to CoQ's ability to easily oxidize and reduce in transporting electrons, it is also adapt at behaving as an antioxidant, preventing lipid peroxidation within the IMM (Mellors and Tappel, 1966b). Endogenous CoQ is decreased in the renal cortex in DN (Sourris *et al.*, 2012), and it has been the subject of several nephropathy studies to date through the development of mitoquinol (MitoQ) (Chacko *et al.*, 2010; Mukhopadhyay *et al.*, 2012). MitoQ is a mitochondria-targeted compound based on the CoQ antioxidant conjugated to a triphenylalkylphosphonium cation (TPP⁺) that is dependent on $\Delta\Psi_m$ to be taken up into the mitochondrial matrix (Murphy and Smith, 2007). MitoQ has been shown to be renoprotective in Ins2-Akita mice (reminiscent of type 1 diabetes), improving tubular and glomerular function, decreasing albuminuria, normalizing glomerular basement membrane thickening and tubulointerstitial fibrosis (Chacko *et al.*, 2010). MitoQ also blocked phospho-Smad2/3-mediated signalling in the kidney, directly implicating ROS derived from mitochondria as a crucial mediator of renal fibrosis in DN. MitoQ may therefore prove to be a suitable tool for regulating mitophagy activity.

Mitophagy and programmed cell death: dangerous liaisons

When considering the potential of exploiting mitophagy in therapeutic strategies, one must remember that the pathways that regulate this paradigm are intricately linked to programmed cell death. Much evidence has emerged that apoptosis and autophagy pathways share common proteins (Figure 3). There is also much evidence to suggest that in the absence of apoptosis, overactivation of autophagy can elicit cell death in mammalian systems (Chen *et al.*, 2007; Oppenheim *et al.*, 2008; Elgendy *et al.*, 2011; Higgins *et al.*, 2011). However, it is unresolved as to whether ACD is physiologically relevant *in vivo*. Yet, whether ACD exists in an

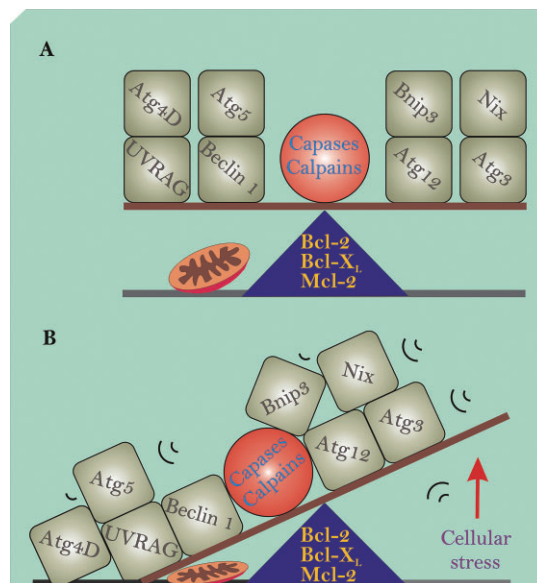


Figure 3

Mitophagy players in life and death. (A) Depicts key autophagy proteins in perfect balance allowing the maintenance of mitochondrial homeostasis. (B) However, with increased cellular stress (e.g. glucose, reactive oxygen species), the balance can be tipped, resulting in some of these proteins being activated by proteases, such as caspases and calpains, whereas others interact with Bcl-2 family members through BH3-only domains.

intrinsic physiological setting becomes irrelevant when pharmacological intervention is brought into the milieu, given that the manipulation of any molecular pathway no longer renders the situation biologically innate. This section will look at the potential caveats of targeting mitophagy in cells that are already under severe stress.

Many of the key autophagic proteins that have been implicated in programmed cell death are involved in regulating mitochondrial turnover. Atg5 was the first Atg protein to be identified to have a role in cell death (Yousefi *et al.*, 2006). Atg5 was shown to be cleaved by calpain-1 or calpain-2, with cleaved Atg5 translocating to the mitochondria where it associated with Bcl-X_L before enacting cyt *c* redistribution and caspase activation. Similarly, Beclin-1 was also reported to become cleaved by caspases at the C-terminus, converting it from a pro-autophagy protein to a pro-apoptotic protein (Wirawan *et al.*, 2010). Once cleaved, Beclin-1 translocates to the mitochondria where it induces the release of cyt *c* and HtrA2/Omi from the IMM (Wirawan *et al.*, 2010). In addition, Beclin-1 also possesses a BH3-only domain and is thought to mediate binding with anti-apoptotic proteins Bcl-2 and Bcl-X_L (Liang *et al.*, 1998; Maiuri *et al.*, 2007; Oberstein *et al.*, 2007). Binding of Beclin-1 with Bcl-2 and Bcl-X_L inhibits autophagy function (Pattingre *et al.*, 2005). UVRAG (ultraviolet irradiation resistance-associated gene), a key regulator of autophagy activity, is also capable of regulating apoptosis via mitochondria through the regulation of Bax (Yin *et al.*, 2011). The interaction between UVRAG and Bax inhibits apoptosis, preventing the induction of the intrinsic apoptotic pathway.

Additionally, several studies have also reported varying roles for the ubiquitin-like modifier Atg12 in mediating mitochondria homeostasis and involvement in cell death (Radoshevich *et al.*, 2010; Rubinstein *et al.*, 2011). Atg12 conjugation with Atg3 has been shown to regulate mitochondrial mass, mitochondrial fusion and cell death involving mitochondria via Bcl-X_L (Radoshevich *et al.*, 2010). This differs greatly from its role in autophagy, where Atg12 conjugates with Atg5 to mediate phosphatidylethanolamine lipidation of LC3 (Hanada and Ohsumi, 2005). Atg12 is further implicated in cell death through a BH3-like domain that allows it to bind with Bcl-2 family members (Rubinstein *et al.*, 2011). This action of Atg12 is independent of conjugation with Atg5 or Atg3, with inhibition of Bcl-2 and Mcl-1 triggering Bax activation and cyt *c* release (Rubinstein *et al.*, 2011). Atg5, Atg12 and Beclin-1 have all been reported to be cleaved during cisplatin-induced AKI in renal proximal tubule cells, which was attenuated by the pan-caspase inhibitor z-VAD-fmk (Herzog *et al.*, 2012). Given that z-VAD-fmk can inhibit other proteases such as calpains and cathepsins, this result in cisplatin-induced injury could be the effect of caspase or calpain activation (Rozman-Pungercar *et al.*, 2003; Bizat *et al.*, 2005). Taken together, these results suggest that crosstalk between autophagy/mitophagy and apoptotic cell death pathways is invoked in acute renal injury and that these interactions should be the focus of future studies on DN.

The mitophagy adaptor proteins Bnip3 and Nix were both initially discovered as pro-apoptotic BH3-only proteins (Chen *et al.*, 1997; 1999). Unlike other BH3-only proteins, neither Bnip3 nor Nix rely on the BH3-only domain to interact with anti-apoptotic Bcl-2 or Bcl-X_L. Rather, it is the C-terminal that is indispensable for binding with these Bcl-2 family members and for mitochondrial translocation (Chen *et al.*, 1999; Ray *et al.*, 2000). A further distinction from other BH3-only proteins lies with their complex roles in cell death, with Nix (Chen *et al.*, 2010) and Bnip3 (Vande Velde *et al.*, 2000; Quinsay *et al.*, 2010; Chavez-Valdez *et al.*, 2012) having dual roles being involved in both apoptosis and programmed necrosis. In cardiomyocytes, Nix is capable of mediating MOMP and apoptosis via a Bax/Bak-dependent pathway, as well as initiating the MPTP for programmed necrosis (Chen *et al.*, 2010). However, Nix evokes programmed necrosis by localizing to the ER where it induces Ca²⁺ release, which is taken up by mitochondria stimulating the MPT (Diwan *et al.*, 2009; Chen *et al.*, 2010).

Bnip3 has been shown to activate during ischaemia-reperfusion injury or hypoxia in various cell systems (Vande Velde *et al.*, 2000; Kubasiak *et al.*, 2002; Kubli *et al.*, 2008; Chavez-Valdez *et al.*, 2012). Like Nix, Bnip3 localizes at both the mitochondria and the ER to regulate apoptosis and programmed necrosis (Zhang *et al.*, 2009; 2010; Landes *et al.*, 2010; Qi *et al.*, 2012). However, the mechanism involving Bnip3 in these cell death pathways has not been as succinctly defined as with Nix. Nonetheless, evidence suggests that when Bnip3 translocates to the OMM, it induces cell death by inducing MPT via a mechanism that is independent of Cyp-D, dissipating $\Delta\Psi_m$ and inducing necrotic cell death (Quinsay *et al.*, 2010). Interestingly, the induction of MPT by Bnip3 appears to be dependent on Bax and Bak (Kubli *et al.*, 2007). A further role for Bnip3 in cell death includes inducing mitochondrial fragmentation via interaction with OPA1

(Landes *et al.*, 2010). In addition, Bnip3 and AIF have been shown to cooperate to induce apoptosis in embryonic epithelial morphogenesis under hypoxic conditions (Qi *et al.*, 2012).

Of particular interest was the discovery that Atg4D can be cleaved by caspase-3 at its N-terminus to reveal a latent mitochondrial leader sequence (Betin and Lane, 2009; Betin *et al.*, 2012). Mitochondrial recruitment of cleaved Atg4D precedes apoptosis induction and is imported into the matrix where it undergoes further processing (Betin *et al.*, 2012). Atg4D recruitment to mitochondria appears to be highly specific, with H₂O₂ inducing Atg4D translocation, an effect not observed during starvation or when using the apoptotic inducer staurosporine (Betin and Lane, 2009; Betin *et al.*, 2012). Notwithstanding, caspase cleavage of Atg4D was shown to be non-essential for mitochondrial recruitment and apoptosis can occur independently of Atg4D translocation (Betin and Lane, 2009). Although, overexpression of cleaved Atg4B did sensitize the cells towards apoptosis (Betin *et al.*, 2012). Further confounding its role in cell death is the fact that Atg4D has a BH3-only domain at its C-terminus, which appears to be required for cell death to occur (Betin and Lane, 2009). Compared to the other autophagins, Atg4C is the only other member to possess a DEVD, caspase-3 cleavage site and mitochondrial leader sequence, although it does not possess a BH3-only domain and its function remains undefined (Betin *et al.*, 2012).

Cleaved Atg4D has a specificity for GABARAP-L1 delipidation, restricting GABARAP-L1 autophagosome formation, suggesting it also has a role in autophagy regulation (Betin and Lane, 2009). Evidently, each Atg4 family member possesses a unique specificity to Atg8 family members and has distinct roles in autophagy. In addition, it has been reported that Atg4A can be cleaved at the C-terminus by caspase-3 and centrally by calpain-1, with the later protease also capable of cleaving Atg4B at the C-terminus (Norman *et al.*, 2010). A role for each of these autophagins in cell death *in vivo* has yet to be established. However, it is apparent that proteases are a key determinant in deciding how Atg4 family members are recruited post-transcription.

The importance of autophagy/mitophagy in renal injury

Autophagy has been implicated in the pathogenesis of a number of diseases such as cancer and neurodegenerative diseases (recently reviewed by Choi *et al.*, 2013). There is a growing body of evidence to indicate that autophagy is linked to the pathogenesis of renal diseases such as DN, AKI and polycystic kidney disease (Huber *et al.*, 2012). Autophagy may have a protective role in renal injury as shown by studies using conditional-knockout mice of the autophagy-related genes *Atg5* or *Atg7*, in the proximal tubule (Kimura *et al.*, 2011; Jiang *et al.*, 2012; S. Liu *et al.*, 2012; Takahashi *et al.*, 2012). *Atg5* and *Atg7* are both required for the elongation and expansion of the isolation membrane during the formation of the autophagosome (Yang and Klionsky, 2010a,b). Knockdown of *Atg5* or *Atg7* sensitizes the kidneys to ischaemia-reperfusion injury (Kimura *et al.*, 2011; Jiang *et al.*,

2012; S. Liu *et al.*, 2012) or to nephrotoxic drugs such as cisplatin (Periyasamy-Thandavan *et al.*, 2008; Yang C. *et al.*, 2008; Takahashi *et al.*, 2012) resulting in an increase in tubular damage, blood urea nitrogen, serum creatinine levels and cell death. Moreover, several of these studies also reported that the accumulation of damaged mitochondria was exacerbated when autophagy was impaired (Kimura *et al.*, 2011; S. Liu *et al.*, 2012; Takahashi *et al.*, 2012).

There is a growing body of evidence that autophagy or mitophagy is altered in DN. An early study in diabetic rats found an association between decreased autophagic activity, as demonstrated by a decrease in volume and densities of autophagic vacuoles in PTECs detected by electron microscopy (Barbosa Junior Ade *et al.*, 1992). The caveat to this study was that the rodents were studied only three days after injection with STZ, in which there is likely to be acute effects of STZ that are unrelated to diabetes (Tay *et al.*, 2005). A similar study showed that regulation of blood glucose with insulin treatment led to the normalization of the density of autophagic vesicles, suggesting that this phenomenon was glucose-mediated (Han *et al.*, 1992). However, insulin itself has been shown to promote mTOR signalling, which negatively regulates autophagy activity (Dennis *et al.*, 2011) (Figure 2), although the sensitivity of this effect can be tissue specific (Naito *et al.*, 2013). Other reports have found markers of autophagy to be increased in the kidney in DN. In particular, Beclin-1 was increased 8 weeks after STZ injection in rats (Wu *et al.*, 2011). Recent studies reveal an up-regulation of p62/SQSTM1, a marker of impaired autophagy, in the kidneys of mice with STZ-induced diabetes (Vallon *et al.*, 2013) and Wistar fatty (fa/fa) rats (Kitada *et al.*, 2011), a model of type 2 diabetes. In glomerular lysates of STZ diabetic mice, LC3-II, Beclin-1 and Atg12-Atg5 levels were decreased over the course of DN (Fang *et al.*, 2013b). Importantly, in that paper, decreased immunofluorescence staining for LC3 was shown in glomerular podocytes within human renal biopsies obtained from patients with DN. Furthermore, *in vitro* studies have demonstrated that high glucose can induce LC3-II and Beclin-1 in podocytes (Ma *et al.*, 2013). Less is known about mitophagy in DN. In a rat model of STZ diabetes, in early diabetes (four weeks after diabetes induction), PINK1 protein was increased in the renal cortex (Smith *et al.*, 2012a). In the same study, up-regulation of PINK1 was not observed in diabetic rats treated with insulin, suggesting that activation of mitophagy was glucose-driven. It is conceivable that early in diabetes, the kidney activates mitophagy to clear dysfunctional mitochondria, but as DN progresses, this process becomes overwhelmed, leading to impairment of mitophagy, accumulation of fragmented mitochondria and cell death. This concept, including the molecular mechanisms that may regulate changes in mitophagy, is discussed in more detail in the subsequent sections.

Several mechanisms have been postulated whereby autophagy could modulate renal fibrosis (Wang and Choi, 2013). In response to injury, glomerular mesangial cells proliferate and produce ECM leading to the development of glomerulosclerosis and renal fibrosis. Collagens are the primary constituents of the ECM. A recent study identified a novel role for autophagy in regulating ECM deposition in mesangial cells by promoting the degradation of intracellular collagen-1 (Kim *et al.*, 2012). Kidneys from mice deficient

in the autophagic protein Beclin-1 exhibited a profibrotic phenotype, with increased collagen-I deposition. In mesangial cells treated with bafilomycin A1 (an inhibitor of autolysosomal protein degradation) increased collagen-1 accumulation and co-localization of collagen-1 with LC3, or LAMP-1 (a lysosome marker) was observed. Conversely, treatment with trifluoperazine, an inducer of autophagy, resulted in decreased TGF- β 1-induced Collagen accumulation. Previous studies have demonstrated the ability of TGF- β 1 itself to induce autophagy in renal mesangial cells. A study by Ding *et al.* showed that TGF- β 1 up-regulated LC3 via the Akt pathway (Ding *et al.*, 2010b). Kim *et al.* demonstrated that TGF- β 1 induces autophagy in mesangial cells via the TAK1-MKK3-p38 signalling pathway and concluded that the dual functions of TGF- β 1, as both an inducer of Col-1 synthesis and an inducer of autophagy and Col-1 degradation underscore the pleiotropic nature of TGF- β 1 (Kim *et al.*, 2012). These findings suggest that autophagy is a cytoprotective mechanism to negatively regulate and prevent excess collagen accumulation in the glomeruli (Wang and Choi, 2013). In the light of these studies, it is plausible that an impairment in autophagic flux in DN would lead to a failure in the clearance of ECM, contributing to enhanced renal fibrosis.

The loss of podocytes, which form the filtration barrier of glomeruli, is considered a key feature of progressive glomerular disease. Within the podocyte, a post-mitotic cell type, a high level of constitutive autophagy has been detected (Hartleben *et al.*, 2010). Podocyte-specific deletion of Atg5 leads to glomerulopathy in ageing mice that is accompanied by an accumulation of oxidized and ubiquitinated proteins, ER stress, proteinuria, podocyte loss and glomerulosclerosis. Moreover, activation of autophagy has been observed in glomeruli from mice with induced proteinuria and in glomeruli from patients with acquired proteinuric diseases (Hartleben *et al.*, 2010). These findings underscore the significance of autophagy as a key homeostatic mechanism to maintain podocyte integrity (Hartleben *et al.*, 2010). Several further studies using rodent models with podocyte-specific deletion of genes encoding proteins involved in autophagy have supported a critical role for autophagy in the maintenance of renal function (Cina *et al.*, 2012b; Bechtel *et al.*, 2013). Interestingly, angiotensin II promotes autophagy in podocytes in an mROS-dependent manner (Yadav *et al.*, 2010).

As discussed earlier, there is some evidence that autophagy may also mediate tubulointerstitial fibrosis. In addition, in tubule-specific TGF- β 1 overexpressing mice, tubular cells were dedifferentiated and finally decomposed by autophagy, with evidence of tubulointerstitial fibrosis, but not epithelial-to-mesenchymal transition, nor was there any sign of apoptosis, suggesting that autophagy might mediate tubular cell death (Koesters *et al.*, 2010).

A deficiency in mitophagy induces oxidative stress in kidneys, as mitophagy blockade leads to the accumulation of damaged mitochondria. In the context of a healthy cell, mitophagy is also a critical negative regulator of inflammatory activation. Damaged mitochondria promote mROS with subsequent mtDNA release and activation of the NLRP3 inflammasome promoting microinflammation (Naik and Dixit, 2011).

Pharmacological targeting of autophagy and mitophagy

As autophagy/mitophagy is either activated or impaired in a variety of pathological processes, pharmacological approaches to promote or to inhibit these pathways are receiving considerable interest (Rubinsztein *et al.*, 2012) and highlight the translational potential of this area of research. Previous studies indicate that impairment of autophagy is a feature of DN, although mitophagy/autophagy may be stimulated in the kidney early in diabetes. Indeed, defects in autophagy and/or mitophagy can occur at different stages of a disease process, which will affect any treatment strategies. Various compounds have been identified as modulating the autophagy/mitophagy process including sirolimus (rapamycin), an mTORC1 inhibitor that inhibits autophagy. The benefits of rapamycin in treating DN have been supported by experimental studies that have shown that inhibition of mTOR activation protects against renal hypertrophy (Sakaguchi *et al.*, 2006), glomerulosclerosis and proteinuria (Godel *et al.*, 2011; Inoki *et al.*, 2011), and mesangial expansion (Lloberas *et al.*, 2006; Yang *et al.*, 2007; Mori *et al.*, 2009) in rodent models of diabetes.

However, in the nephrology field, rapamycin is best known as an immunosuppressant commonly used during kidney transplantation and has side effects that are limiting to therapy (Morath *et al.*, 2007). Moreover, kidney transplant patients that are treated with rapamycin are more susceptible to new-onset diabetes (Teutonico *et al.*, 2005; Johnston *et al.*, 2008). The differences between experimental and clinical findings may lie in results from recent studies that suggest that rapamycin, once considered to be exclusively an mTORC1 inhibitor, can in fact inhibit mTORC2 under more chronic treatment regimes. Recently, rapamycin through chronic inhibition of mTORC2 was shown to impair glucose tolerance and insulin activation in *in vivo* mouse studies akin to type 2 diabetes (Lamming *et al.*, 2012). mTORC2 differs from mTORC1 in that it is regulated by rictor (rapamycin-insensitive companion of mTOR), whereas mTORC1 is regulated by raptor (rapamycin-associated protein of mTOR). The emerging function of mTORC2 appears to be regulating autophagy gene expression through acting on Akt, which phosphorylates FOXO3a (Mammucari *et al.*, 2007; Zhao *et al.*, 2007) (Figure 2). In muscle cells, some of the autophagy genes that FOXO3a regulates include LC3, Bnip3, Nix (Mammucari *et al.*, 2007), Atg4b and Atg12l (Zhao *et al.*, 2007), all of which play key roles in mitophagy. These findings may help to explain the adverse effect of conditional knockout of mTOR in podocytes, which disrupted autophagic flux and led to renal failure 5 weeks after birth (Cina *et al.*, 2012a).

The mTOR signalling pathway evidently contributes to the impairment of autophagy during DN. However, the above studies suggest that rapamycin or similar drugs, such as rapalogs (derivatives of rapamycin) or dual mTOR inhibitors like the chemotherapeutic drug AZD8055 (Huang *et al.*, 2011) may not be suitable for the long-term stimulation of autophagy required for a chronic treatment regime, such as in people with DN. Recently, rilmenidine, a clinically approved anti-hypertensive drug was demonstrated to stimulate

autophagy activity via an mTOR-independent pathway (Rose *et al.*, 2010). Rilmenidine is an imidazoline receptor 1 agonist that has been shown to attenuate polyglutamine toxicity in the brain, through increased autophagy in a Huntington's disease mouse model (Rose *et al.*, 2010). Rilmenidine has been shown to bind to imidazoline receptor 1 in the proximal tubule (Lachaud *et al.*, 1989) and to reduce microalbuminuria in patients with type 2 diabetes and hypertension (Reid, 2000). Although activation of autophagy via mTOR signalling is a non-selective means of removing dysfunctional mitochondria, this pathway may be important in modulating mitophagy activity. However, drugs such as rilmenidine (Renna *et al.*, 2010), which bypass mTOR and are not nephrotoxic may be required to promote autophagy activity in DN.

Because mitophagy is dependent on mitochondrial dynamics, modulation of fission and fusion may target the process of mitophagy. It is interesting to note that in AKI, treatment of mice with mdivi-1, a Drp1 inhibitor (Cassidy-Stone *et al.*, 2008), was renoprotective in an ischaemia-reperfusion injury model; however, mdivi-1 only partially suppressed ischaemia-induced mitochondrial fragmentation in proximal tubular cells, from 43 to 32% (Brooks *et al.*, 2009). Treatment with mdivi-1 reduced serum creatinine and blood urea nitrogen, tubular damage and apoptosis, but these studies were performed in an acute setting. Preclinical studies are required to investigate efficacy and possible adverse effects in a chronic setting such as DN.

The relationship between ROS production and removal must be delicately balanced in order to stimulate mitophagy activity (Frank *et al.*, 2012). Too much ROS is detrimental, but too little can result in disruption to redox signalling. This paradox may explain the lack of efficacy of antioxidant therapies to date (Benfeito *et al.*, 2013), with other sources of ROS also needing to be considered when treating DN (Forbes *et al.*, 2008; Singh *et al.*, 2011). To this end, the development of mitochondrial targeted antioxidants may be beneficial for controlling mROS production, while minimizing interference with cellular redox signalling. However, the benefits of using antioxidants of this nature need context. Although mitochondria-targeted antioxidants may limit oxidative damage and be capable of repairing lipid membranes, they may not restore functionality to damaged mitochondria (e.g. repair damaged proteins or mtDNA). The balance of healthy mitochondria within a cell may only be restored by removal of damaged mitochondria through mitophagy. It is plausible that antioxidants may also target the process of mitophagy. A previous study showed that treatment with CoQ decreased both mitochondrial fragmentation and size (Persson *et al.*, 2012), indicating that mitochondrion targeted antioxidant treatment may protect against altered morphology and function in diabetes, and indeed may modulate mitophagic flux.

Distinct from MitoQ, other forms of mitochondria-targeted antioxidants known as SS-peptides that are not dependent on $\Delta\Psi_m$ have also been developed (Szeto, 2008). These cationic antioxidants can passively diffuse into the mitochondria and reside in the IMM. Mitochondria-targeted antioxidant SS-31 has been demonstrated to be beneficial in AKI studies involving ischaemic-reperfusion injury and unilateral ureteral obstruction (Mizuguchi *et al.*, 2008; Szeto *et al.*, 2011; Birk *et al.*, 2013). However, it remains to be

established whether these antioxidants will be equally beneficial in more chronic nephropathy settings such as DN, particularly in situations where treatments more often than not will need to be administered following the diagnosis of the onset of the disease. Furthermore, in the case of mitophagy regulation, where $\Delta\Psi_m$ and ROS production are considered critical signals for the turnover of damaged mito-

chondria, TPP⁺ compounds might be more advantageous than SS-peptides. In manipulating mitophagy activity, preferential antioxidant treatment of healthy or partially damaged mitochondria with $\Delta\Psi_m$ would minimize damage and oxidative stress production, allowing autophagosomes to target damaged mitochondria (without $\Delta\Psi_m$ and producing oxidative stress). In chronic situations where mitophagy

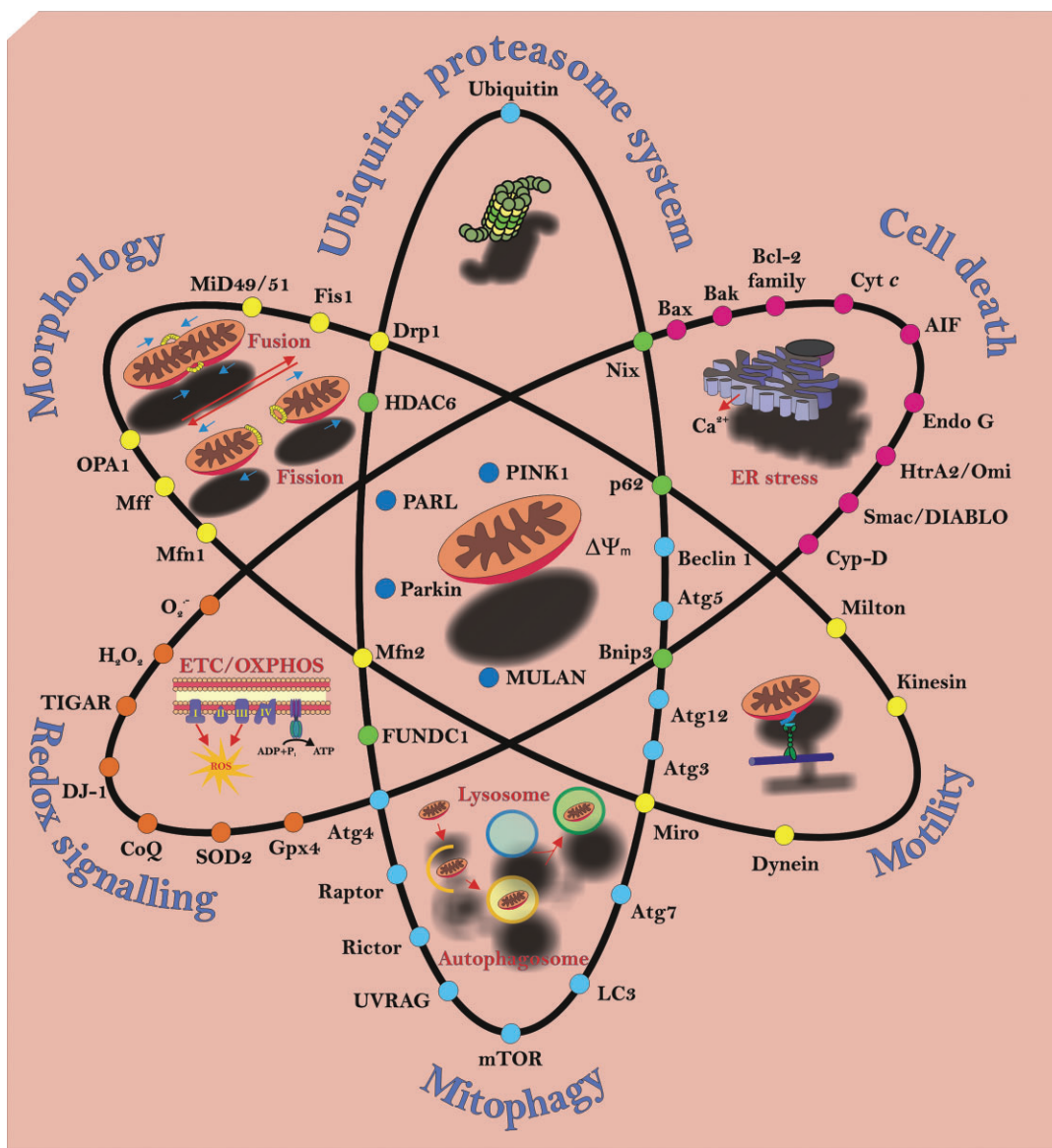


Figure 4

Balancing mitochondrial homeostasis. This schematic illustrates the interaction between mitochondrial biogenesis, dynamics, organelle turnover and cell death, in addition to the major players involved in these processes. Mitochondria biogenesis involves oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC). This process generates energy for the cell in the form of ATP, but in the process also generates reactive oxygen species (ROS). These ROS are involved in redox signalling regulated by antioxidants and transcription factors (orange circles). Mitochondria are dynamic organelles that are in constant motion, being transported around the cytoplasm while undergoing changing morphology (yellow circles). When mitochondria become dysfunctional via oxidative stress, mitochondrial dynamics is arrested and the ubiquitin proteasome system and mitophagy come into play to turnover the damaged organelles (pale blue circles). Central in regulating this process is the interaction between PARL (presenilin-associated rhomboid-like protease), PINK1 and Parkin (dark blue circles), and the involvement of adapter proteins (green circles). When cellular stress (e.g. damage to mitochondria and ER stress) overwhelms cellular homeostasis, cells can undergo diverse forms of death. Depending on the type and severity of the stress, mitochondria can regulate multiple cell death pathways involving several different signalling pathways (pink circles).

machinery may be overwhelmed by the magnitude of dysfunctional mitochondria, enhanced preferential selection of these organelles would be a considerable advantage. Although SS-peptides act independently of $\Delta\Psi_m$, the rate of mitophagy activity may be compromised with blanket suppression of ROS masking redox signalling from damaged mitochondria.

Closing comments

Although there is ample evidence that there is a disturbance of mitochondrial bioenergetics in the kidney in DN, it is clear that there is a lack of studies characterizing the role of mitophagy in this disease process. How the process of mitophagy is changed in DN and if this change is beneficial or detrimental to kidney function still remains to be fully understood. What we know of mitophagy is drawn from studies across many mammalian cellular systems. From these studies, it is apparent that mitochondrial homeostasis is a tightly regulated balance between several different processes that are responsible for maintaining dynamics, motility, redox signalling and, ultimately, the decision between life and death. Mitophagy and the UPS are intricately intertwined with each of these processes to ensure a healthy pool of mitochondria is maintained (Figure 4). Yet, it is the mode and severity of the insult as well as unique cellular characteristics (e.g. presence of SGLT2, GLUT2 and Na⁺/K⁺-ATPase transporters to facilitate the glucose gradient and relatively rich mitochondria content in PTECs) that will influence the type of mitophagic response. Thus, underscoring the need to elucidate the mechanisms that regulate mitophagy in the kidney.

Much attention has been given to studying mitochondrial dysfunction in DN, raising the prospects of pharmacological agents that may impede progression of the disease. However, DN diagnosis usually occurs once the onset of the disease is well advanced. Strategies to impede mitochondrial dysfunction may not be sufficient to restore kidney homeostasis and only delay the progression of DN. Despite the lack of studies focused on mitophagy in DN, it stands to reason that this process may be implicated in its pathology. The accumulation of dysfunctional mitochondria in DN is evidence of either an impairment of mitophagy, or an inability of this process to meet the influx of damaged mitochondria. An improved understanding of the mechanisms that regulate mitophagy may lead to the discovery of new targets for therapeutic approaches of DN that can prevent or reverse pathology. Drug screening for agents that interact with mitophagy regulators could provide further therapeutic targets. Therapeutic strategies that promote mitophagy activity offer the prospect of not only eliminating dysfunctional mitochondria, but also the possibility of restoring mitochondrial biogenesis.

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Conflict of interest

None.

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