Role of HDAC6 in Transcription Factor EB Mediated Clearance of Misfolded Proteins in Chronic Kidney Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Institute of Medical Science, University of Toronto

2017

Abstract

The autophagy-lysosomal pathway is a homeostatic mechanism to prevent the accumulation of misfolded proteins. Here, we observed a downregulation in a master regulator of autophagy, transcription factor EB (TFEB) and an increase in misfolded protein accumulation in kidneys from humans with diabetic kidney disease and in subtotally nephrectomized (SNx) rats, pointing to dysregulated autophagy as a common occurrence in chronic kidney disease (CKD). In assessing methods to induce autophagy, we found that inhibition of histone deacetylase 6 (HDAC6) caused hyperacetylation and nuclear translocation of TFEB, and reduced cell death in cultured proximal tubule cells. Similarly, in SNx rats, HDAC6 inhibition decreased misfolded protein accumulation in tubule epithelial cells, attenuated tubule cell death, diminished fibrosis and blunted proteinuria. These findings point to the occurrence of dysregulated autophagy in CKD and identify HDAC6 inhibitors as a novel method to activate TFEB mediated upregulation of the autophagy-lysosomal pathway that may confer renoprotective benefits.
I would like to sincerely thank my supervisor and mentor Dr. Andrew Advani, for his guidance, constructive feedback and patience during my time as a student. I would like to thank him for giving me the opportunity to grow, contribute to the wealth of knowledge in the lab and to collaborate with my fellow trainees. I would also like to thank him for teaching me strategies in resilience and for his words of encouragement during times of adversity. Beyond our time in the lab, I would like to thank him for his excellent career advice and for the opportunity to shadow him in the clinic. Dr. Advani’s approach to medicine, both as a provider and an innovator, has inspired me to pursue a similar career path. I am truly grateful to have found such an important mentor.

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It is a pleasure to thank our collaborators, Dr. Laurette Geldenhuys and Dr. Ferhan S. Siddiqi for providing samples for our human correlative studies, and for their feedback on our manuscript of this work.

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I would like to dedicate this thesis to my supportive parents, Jay Brijmohan and Shanta Brijmohan, my twin sister Amanda Brijmohan, and to my late grandparents Ramdei Brijmohan and Ragnauth Bridgemohan, for always encouraging me to be resolute in the pursuit of my goals.
Contributions

Dr. Laurette Geldenhuys and Dr. Ferhan S. Siddiqi provided archival human kidney samples for human correlative studies (Figure 1 and Figure 2).

Dr. Golam Kabir performed the rat sham and subtotal nephrectomy surgeries throughout this study.

Bridgit B. Bowskill contributed to the assessment of proteinuria (Figure 16), systolic blood pressure, glomerular filtration rate, body weight and kidney weight and general conductance of the interventional study (Table 1).

Suzanne L. Advani contributed to the preparation of tissue for immunohistological stains for p62 in human kidney sections (Figure 2) and collagen IV in rat kidney sections (Figure 17).

Dr. Youan Liu contributed to the isolation of RNA from paraffin-embedded human kidney sections (Figure 1) and maintenance of NRK-52E cells for in-vitro experiments.

Dr. Syamantak Majumder contributed to assessment of TFEB mRNA levels in human kidney samples (Figure 1) and preparation of samples for immunoprecipitation with TFEB (Figure 10).

Sarah McGaugh contributed to the quantification of nuclear TFEB in NRK-52E cells (Figure 11).

Dr. Sri N. Batchu contributed to the assessment of cell death in-vitro (Figure 12-13) and to the nuclear fractionation procedure used to assess nuclear TFEB levels in kidney homogenates (Figure 18).

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<th>Description</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-Amino-actinomycin</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>AGES</td>
<td>advanced glycated end products</td>
</tr>
<tr>
<td>ACEi</td>
<td>angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>ARB</td>
<td>angiotensin receptor blocker</td>
</tr>
<tr>
<td>ALP</td>
<td>autophagy-lysosomal pathway</td>
</tr>
<tr>
<td>ATGs</td>
<td>autophagy-related proteins</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>PB1</td>
<td>Bem1p</td>
</tr>
<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CIHI</td>
<td>Canadian Institute for Health Information</td>
</tr>
<tr>
<td>CORR</td>
<td>Canadian Organ Replacement Register</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>chromatin immunoprecipitation sequencing</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CLEAR</td>
<td>Coordinated Lysosomal Expression and Regulation network</td>
</tr>
<tr>
<td>DD</td>
<td>deacetylase domains</td>
</tr>
<tr>
<td>DiaComp</td>
<td>Diabetic Complications Consortium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMB</td>
<td>dynein motor binding domain</td>
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ER: endoplasmic reticulum
ESRD: end-stage renal disease
ECL: enhanced chemiluminescent substrate
eGFR: estimated glomerular filtration rate
eIF2α: eukaryotic initiation factor 2 alpha
FBS: fetal bovine serum
FOXO: Forkhead box O
GATA-1: GATA binding factor 1
GATA4: GATA binding protein 4
GFR: glomerular filtration rate
GEF: guanine nucleotide exchange factor
HSP90: heat shock protein 90
HSF1: heat-shock transcription factor 1
HATS: histone acetyltransferases
HDAC: histone deacetylase
HDAC6: histone deacetylase 6
IMPC International Mice Phenotyping Consortium
IRE1α: inositol-requiring enzyme 1
KDOQI: Kidney Disease Outcomes Quality Initiative
Klf-4: Kruppel-like factor 4
LcoR: ligand-dependent corepressor
LAMP1: lysosomal associated membrane protein 1
LSD: lysosomal storage disorder
mTORC1: mammalian target of rapamycin complex 1
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>LC3</td>
<td>microtubule associated protein 1A/1B light chain 3B</td>
</tr>
<tr>
<td>MCOLN1:</td>
<td>mucolipin 1</td>
</tr>
<tr>
<td>NES:</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NF-κB:</td>
<td>nuclear factor kappa-light-chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NLS:</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NRK:</td>
<td>normal rat kidney</td>
</tr>
<tr>
<td>sequestosome 1:</td>
<td>p62/SQSTM1</td>
</tr>
<tr>
<td>PBS:</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PS:</td>
<td>phospholipid phosphatidylinerine</td>
</tr>
<tr>
<td>PERK:</td>
<td>protein kinase R-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>Rags:</td>
<td>Rag GTPases</td>
</tr>
<tr>
<td>RT-PCR:</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>RIN:</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>ACY-1215</td>
<td>rocilinostat</td>
</tr>
<tr>
<td>RUNX2:</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>SE14:</td>
<td>Ser-Glu-containing tetrapeptide</td>
</tr>
<tr>
<td>siRNA:</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SGLT2</td>
<td>sodium glucose cotransporter 2</td>
</tr>
<tr>
<td>s.c:</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SBP:</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>TUNEL:</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>TFEB:</td>
<td>transcription factor EB</td>
</tr>
<tr>
<td>TGF-β:</td>
<td>transforming growth factor-β</td>
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TBS-T: tris-buffer saline-tween 20
ZnF-UBP or BUZ: ubiquitin binding zinc finger domains
UPS: ubiquitin proteasome system
UPR: unfolded protein response
UOO unilateral ureteral obstruction
v-ATPase: vacuolar-type H+-ATPase
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- 10X Running Buffer
- 10X TBS Buffer
- 5% Blocking Solution (for Immunoblot)
- 2% Blocking Solution (for Immunofluorescence)
- Citric Acid Buffer
- Scott’s Tap Water
- Homogenization Buffer
- 5% FITC-inulin
Chapter 1
Literature Review

Sections have been modified from Batchu SN*, Brijmohan AS*, Advani A [2016]. The Therapeutic Hope for HDAC6 Inhibitors in Malignancy and Chronic Disease. Clinical Science 987-1003. *These authors contributed equally to this publication.

1 Chronic Kidney Disease: Scope of the Problem

Chronic kidney disease (CKD) can be a devastating condition that shortens the quality and quantity of life for many Canadians, and its prevalence is increasing at an alarming rate. According to the Canadian Organ Replacement Register (CORR) annual report by the Canadian Institute for Health Information (CIHI), three million Canadians are affected by CKD today, reflecting an increase of 35% over the last decade (CORR, 2015). This increase is associated with a 60% increase in the prevalence of diabetes, named the leading cause of CKD in the industrialized world (CORR, 2015). Presently, the best therapeutic option for patients faced with end-stage renal disease (ESRD), is kidney transplantation, but the demand for kidneys consistently outweighs supply, requiring alternative forms of renal replacement therapy such as dialysis. While a large proportion of patients will ultimately depend on dialysis, this is a time intensive option that costs the Canadian health care system an average of $95,000-$107,000 per person per year (Klarenbach et al., 2014). In addition, mortality for patients on dialysis is high, with less than 45% surviving after five years (CORR, 2015). With serious questions about the economic sustainability of current kidney disease treatments, coupled with a growing margin between kidney supply and demand for transplantation, new therapies must be explored to manage the growing kidney disease burden in an aging Canadian population (CORR, 2015).
CKD encompasses a group of disorders that impair the structure and function of the kidney. This leads to impairment in the kidney’s ability to complete its normal roles of excretion of waste, reabsorption of nutrients, pH and fluid balance, and blood pressure regulation. Impaired function in these roles is captured in laboratory analyses of glomerular filtration rate (GFR) and urine albumin and therefore, these tests are used clinically in determining the presence and severity of CKD. According to the National Kidney Foundation’s KDOQI Guidelines, measures of GFR are used to classify kidney disease into five stages: greater than 90 mL/min per 1.73 m² (stage 1), 60-89 mL/min per 1.73 m² (stage 2), 30-59 mL/min per 1.73 m² (stage 3), 15-29 mL/min per 1.73 m² (stage 4) and less than 15 mL/min per 1.73 m² (stage 5 or ESRD) (National Kidney Foundation, 2002).

In addition to GFR decline, increasing albuminuria levels positively correlate with mortality, worsening kidney outcomes and an increased risk of cardiovascular disease (Astor et al., 2011; de Jong and Curhan, 2006). Over time, trace amounts of albumin, termed microalbuminuria (200 µg/min or 30-300 mg/d), may appear in the urine as a marker of early kidney disease. While very common amongst people with diabetes, with 20-40% of patients experiencing microalbuminuria early in their disease, untreated microalbuminuria can result in macroalbuminuria (urine albumin excretion rate greater than 200 µg/min), and correlates with declining GFR. In addition to a progression to ESRD, declining GFR is associated with a five-fold increased risk of cardiovascular disease relative to the general population (Stenvinkel, 2010). Beyond an increased cardiovascular risk, progressively declining kidney function is also associated with acute kidney injury, infection, cognitive decline, and frailty (Hailpern et al., 2007; James et al., 2010; James et al., 2009), thus further complicating the management of an already complex disease.

In the industrialized world, the major pathological processes leading to CKD are 1) diabetes and 2) hypertensive nephrosclerosis, both of which are associated with diabetes, hypertension,
cardiovascular disease, obesity and old age (Pinto, 2007). With 50% of people with diabetes developing some form of kidney disease, diabetic kidney disease is the most common cause of renal failure (Saran et al., 2016) and is likely related to both local and systemic changes to the renal milieu under hyperglycemic conditions. For example, in terms of its pathophysiology, increased protein and glycated products in the urinary filtrate leads to damage of the tubulointerstitium, the compartment of the kidney that comprises 80% of the renal volume (Remuzzi et al., 2006). Under conditions of deranged hyperglycemic and metabolic conditions, products such as advanced glycated end products (AGEs) can accumulate. In the proximal tubule, reabsorption of AGEs can trigger profibrotic and proapoptotic signalling, the deposition and impaired clearance of fibrotic matrix components such as collagen and a consequent reduction in renal function (Yamagishi and Matsui, 2010). In addition to increased fibrogenic factors, inflammatory cytokines in the renal parenchyma and impaired clearance of matrix proteins also contribute to the manifestation of maladaptive fibrotic remodelling (Hodgkins and Schnaper, 2012).

Irrespective of the primary cause however, research shows that once kidney disease progresses past a critical point, further decline is irreversible and independent of the initial insult, suggesting cellular derangements that may precede the classical pathological changes noted above. In shifting the focus from parenchymal changes to cellular changes, accumulating evidence points to a derangement in the homeostatic capacity of tubule cells to maintain protein folding fidelity under stressful disease conditions (Cybulsky, 2013). This increase in protein misfolding at the site of the endoplasmic reticulum (ER), termed endoplasmic reticulum stress (ER stress), may be a precipitating factor that leads the tubule cell along a slippery slope of maladaptive signaling and
irreversible damage that manifests itself as increased fibrosis and impaired renal function as discussed below.

2 Proteostasis and Kidney Disease

2.1 Overview: Endoplasmic Reticulum Stress and Quality Control

Cells maintain protein homeostasis (proteostasis) through intricate networks of regulation of the endoplasmic reticulum and mitochondria, acting to maintain the fidelity and diversity of the proteome (Balch 2008, Powers 2013). These networks are two-fold. They include i) coordination of chaperones, such as heat shock proteins, to optimize protein folding, and ii) degradation systems for the removal of misfolded, aggregated or dysfunctional proteins. These degradation systems include, initially, the ubiquitin proteasome system (UPS), and then, the autophagy-lysosomal pathway (ALP).

The endoplasmic reticulum has developed mechanisms to sense the accumulation of misfolded proteins, and can impart signalling to the nucleus to increase transcription of chaperones to assist in refolding of individual peptides; an adaptive reaction known as the unfolded protein response (UPR) (Inagi et al., 2014). The presence of misfolded protein in the endoplasmic reticulum signals the release of ER membrane bound binding immunoglobulin protein (BiP), which initiates the UPR pathway through activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1α) and protein kinase R-like endoplasmic reticulum kinase (PERK) signalling (Cybulsky, 2013). While ATF6, and IRE1α increase the transcription of chaperones, PERK signalling leads
to phosphorylation of eukaryotic initiation factor 2 α (eIF2α). Phosphorylation of eIF2α reduces
the rate of translation by inhibiting the formation of the preinitiation complex for translation (de
Haro et al., 1996). This effectively halts the formation of new peptides as chaperones attempt to
refold aberrant proteins in the ER.

When the amount of misfolded proteins surpasses the refolding ability of molecular chaperones
and the UPR, misfolded proteins are shuttled towards the ubiquitin proteasome system (UPS) for
bulk protein degradation (Cybulsky, 2013; Dobson, 2003). In this system, misfolded protein
substrates are covalently tagged with ubiquitin through a three step ubiquitin ligase enzymatic
reaction. The addition of this polyubiquitin tag shuttles misfolded protein to the 26S proteasome,
a barrel shaped structure that recognizes, unfolds and degrades substrates into smaller peptide
fragments (Olzmann et al., 2008).

When the ubiquitin proteasome system fails to contend with the growing number of misfolded
proteins, misfolded proteins are sequestered into large protein aggregates, known as aggresomes,
through the adaptor protein, p62, also known as sequestosome 1 (SQSTM1) (Komatsu and
Ichimura, 2010). As a key signalling molecule in the autophagosome-lysosomal pathway, p62,
along with additional binding partners, sequesters protein aggregates for bulk degradation through
the autophagy-lysosomal pathway through its ubiquitin recognition domain and self-
oligomerization through its N terminal Phox and Bem1p (PB1) domain (Komatsu et al., 2007;
Nezis et al., 2008). During autophagy, under the coordination of autophagy-related proteins
(ATGs), a large, double membrane structure known as the autophagosome develops and
sequesters cytoplasm, organelles and p62-tagged protein aggregates (Komatsu and Ichimura, 2010). Whereas the proteasomal system is dependent on deubiquitination and unfolding of its substrates for degradation, the autophagosome degrades large numbers of protein aggregates through fusion with the lysosome in a pathway known as the autophagy-lysosomal pathway (Mizushima, 2009; Periyasamy-Thandavan et al., 2008). This process is summarized in Figure A.

2.2 Endoplasmic Reticulum Stress in the Pathogenesis of Kidney Disease

Proximal tubule epithelial cells are highly specialized for the reabsorption and secretion of water, solutes and proteins into the filtrate for maintenance of pH and osmotic regulation. They contain extensive endoplasmic reticulum for the management and modification of secreted or reabsorbed products. Because of this role, proximal tubule cells are sensitive to impaired proteostasis and depend heavily on the UPR to ensure proper protein folding. However, states of chronic ER stress can overwhelm the UPR and cause cells to initiate apoptosis (Inagi, 2010; Yamabara et al., 2013), leading to loss of tubule cells and a progression to CKD (Taniguchi and Yoshida, 2015). Known inducers of ER stress in renal tubules include proteinuria (Ohse et al., 2006), hyperglycemia (Lindenmeyer et al., 2008), uremic toxins (Kawakami et al., 2010) and nephrotoxins such as cisplatin (Khan et al., 2013). Not only do these factors increase ER stress, but they also reduce the efficiency of the UPR, resulting in the cellular decision to undergo UPR mediated apoptosis (Inagi et al., 2014). This accelerated rate of tubule cell loss stimulates fibrotic remodelling and a progressive decline in renal function.
In the setting of chronic impairment of proteostasis, it is possible that the apoptotic result of long term ER stress could also result from a dysregulation in autphagic capacity to clear away misfolded proteins. Aberrant autophagy/lysosomal function is a common feature of many non-renal disorders, including lysosome storage disorders (LSD), neurodegenerative disorders, and aging. Since an appropriate autophagic response is necessary to eliminate damaged proteins, these disorders are associated with accumulation of damaged mitochondria and protein aggregates that can impair cell survival (Vitner et al., 2010). However, mechanisms of dysregulated autophagy have yet to be fully defined in the setting of CKD.

3 Regulation of the Autophagy-Lysosomal Pathway

3.1 Overview

Cells have developed mechanisms to upregulate levels of autophagy to meet the demands of stress conditions, such as protein misfolding, oxidative stress and starvation. In addition to post-translational modification of regulatory proteins, several transcription factors play key roles in autophagy activation and/or repression by changing expression levels of proteins involved in the autophagy pathway. For example, transcription factors such E2F1, GATA binding factor 1 (GATA-1) and members of the Forkhead box O (FOXO) family upregulate autophagic processes, while GATA binding protein 4 (GATA4) represses these pathways (Fullgrabe et al., 2014). These transcription factors fine-tune control of different proteins involved in autophagy. Equally important however, is the ability to regulate the lysosome’s capacity to manage increased autophagic activity and degrade autophagic substrates.
Originally identified in the early 1950s, lysosomes are organelles rich in acidic hydrolases that breakdown cytosolic components such as macromolecules and damaged organelles upon fusion of the autophagosome and the lysosome. Whereas it was once believed that lysosomes served a housekeeping, rather, that regulated process, recent evidence shows that under conditions of cellular stress, lysosomal biogenesis can be transcriptionally induced to meet growing degradative demands. In their analysis of the promoter regions of lysosomal genes, Sardiello and colleagues identified a key 10-base-pair motif (GTCACGTGAC) located within 200 base pairs of the transcriptional initiation site of lysosomal genes. This motif contained an E-box (CANNTG) that binds a family of transcription factors known as the MiTF/TFE family. Collectively, this suggested that lysosomal biogenesis can be induced through concurrent upregulation of lysosomal genes to meet the growing demands of the cell. The network of lysosomal genes, under shared transcriptional control, is known as the Coordinated Lysosomal Expression and Regulation network (CLEAR network) (Sardiello et al., 2009). While the previously discussed transcription factors impart fine-tuned control of autophagic proteins, the CLEAR network, as the name suggests, allows for a larger, coordinated response to increase lysosomal biogenesis and the collective activity of the autophagy-lysosomal pathway. Because of this, transcription factors that regulate the CLEAR network have been termed “master regulators” of autophagy (Settembre and Medina, 2015). A transcription factor that has gained a lot of attention as a master regulator is transcription factor EB (TFEB) (Figure A).
3.2 Transcription Factor EB (TFEB): A Master Regulator of the Autophagy-Lysosomal Pathway

TFEB belongs to the MiTF/TFE family of basic helix-loop-helix transcription factors and is related to three additional family members: TFE3, MiTF, and TFEC. While MiTF and TFE3 are not considered major regulators of lysosomal biogenesis (Hershey and Fisher, 2004; Meadows et al., 2007; Motyckova et al., 2001), TFEB is unique in its breadth of lysosomal targets. Overexpression of TFEB induced transcription of multiple lysosomal genes, namely, subunits of the vacuolar-type H+-ATPase (v-ATPase), lysosomal transmembrane proteins such as the lysosomal associated membrane protein 1 (LAMP1), and lysosomal enzymes, indicative of an increase in lysosomal number (Sardiello et al., 2009). Furthermore, genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) found that CLEAR elements in the promoter regions for lysosomal genes were highly enriched with TFEB, suggestive of TFEB’s ability to upregulate large networks of lysosomal genes simultaneously (Palmieri et al., 2011). In addition to lysosomal genes, TFEB can also bind to the promoter regions of genes associated with autophagy, such as beclin-1 (Palmieri et al., 2011). Because of its ability to upregulate both of these degradative pathways, TFEB is now viewed as a master regulator of the autophagy-lysosomal pathway (Settembre et al., 2011).

3.3 Mechanisms of TFEB Activation

Under conditions of cellular stress, cells reduce rates of protein synthesis and increase autophagic breakdown of macromolecules to maintain the supply of ATP and amino acids for new protein synthesis. Cells have developed methods of sensing cellular stress though the activation of
mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates cell division and nutrient management. Under normal conditions, mTORC1 is active and is recruited to the lysosomal surface where it directs normal protein synthesis and prevents autophagy. Under stressed conditions however, mTORC1 is inactivated and thus triggers a cascade of events that stimulate autophagic processes (Raben and Puertollano, 2016). One of these signalling events includes the regulation of TFEB.

Under conditions of nutrient abundance, TFEB is localized to the cytosol where it is recruited to the lysosomal surface. Once there, mTORC1 phosphorylates TFEB at serine 211. Phosphorylation at this site stimulates binding to the cytosolic chaperone 14-3-3, forming a binding complex that sequesters TFEB in the cytosol and prevents its nuclear translocation (Roczniak-Ferguson et al., 2012; Settembre et al., 2012).

This regulation of TFEB is achieved through interactions with the lysosomal surface protein Ragulator, a pentameric protein complex with guanine nucleotide exchange factor (GEF) function (Zoncu et al., 2011). Ragulator interacts with v-ATPases on the lysosomal surface to detect changes in amino acid availability. In addition, Ragulator tethers Rag GTPases (Rags) to the lysosomal surface and regulates its nucleotide state depending on nutrient availability. Under conditions of abundance, Rags will interact with TFEB and recruit mTORC1 to the lysosomal surface thus promoting spatial-temporal colocalization of TFEB and mTORC1 to the outer lysosomal membrane. As a result, mTORC1 can phosphorylate TFEB, promote its interaction with 14-3-3 and maintain TFEB in the cytosol (Zoncu et al., 2011). However, under conditions
of nutrient deprivation, the conformation of Rags proteins change, preventing their interaction with TFEB and mTORC1. This prevents mTORC1 mediated inhibition of TFEB, leading to calcineurin mediated dephosphorylation of TFEB (Medina et al., 2015), release from its binding partner 14-3-3 and its subsequent nuclear translocation. Once in the nucleus, TFEB can upregulate the CLEAR network to increase the activity of the autophagy-lysosomal pathway (Martina et al., 2014). This processed is summarized in Figure A. The essential nature of TFEB in increasing autophagy-lysosomal pathway activity means that it has the potential to clear misfolded proteins efficiently upon induction. Indeed, many researchers have found initial success in inducing TFEB activity in the treatment of model diseases characterized by protein misfolding.
Figure A. Summary of the autophagy-lysosomal pathway. Under conditions of increased endoplasmic reticulum stress, misfolded proteins aggregate and are tagged by the protein aggregate marker p62, marking it as a substrate for autophagy mediated degradation. A double membrane structure known as the autophagosome forms around protein aggregates and fuses with the lysosome for degradation by lysosomal enzymes. The autophagy-lysosomal pathway is regulated by transcription factor EB (TFEB), which can increase the degradative capacity of this pathway as needed. Under basal conditions, mTORC1 phosphorylates TFEB thereby promoting its interaction with its binding partner 14-3-3 and sequestering it in the cytosol. Upon sensing cellular stress, mTORC1 is inactivated, resulting in dephosphorylation of TFEB, its release from 14-3-3 and subsequent nuclear translocation. There, it can upregulate a network of autophagy and lysosomal genes to increase the activity of the autophagy-lysosomal pathway.
3.4 TFEB as a Therapeutic Target

Neurodegenerative Disease

Studies have identified modulation of TFEB activity as a promising therapy for diseases associated with impaired autophagic-lysosomal function. Specifically, in the context of neurodegenerative disease, induction of TFEB activity has been found to improve protein clearance in several models of Alzheimer’s disease, tauopathies and Parkinson’s disease.

Alzheimer’s disease is characterized by the abnormal deposition of protein aggregates known as β-amyloid (Aβ) plaques and neurofibrillary tangles composed of aggregates of phosphorylated tau protein (Himmelstein et al., 2012). This accumulation of protein aggregates has been linked to a dampened autophagic response, as evidenced by a downregulation in TFEB and LAMP1 in brain tissues from Alzheimer’s patients. In aged mice, normalization of TFEB levels decreased Aβ plaques in both astrocytes and neurons, alleviated Aβ pathology and improved cognitive function (Xiao et al., 2015). Similarly, in models of tauopathies, characterized by the abnormal aggregation of neuronal phosphorylated tau protein, overexpression of TFEB increased clearance of phosphorylated tau aggregates (Chauhan et al., 2015).

Parkinson’s disease results from a loss in dopamine producing neurons in the substantia nigra due to the development of α-synuclein aggregates in their cytoplasm. Toxicity of these protein aggregates has been linked to a downregulation of TFEB, resulting in lysosomal depletion and insufficient autophagy (Dehay et al., 2013). As such, pharmacological inhibition of mTORC1
was found to activate residual TFEB, induce its nuclear translocation, improve clearance of α-synuclein aggregates and reduce neurotoxicity (Decressac et al., 2013). Given this success, modulation of TFEB is now being investigated as a therapy for other neurological conformational disorders, namely, spinal and bulbar muscular atrophy and Huntington’s disease (Raben and Puertollano, 2016).

**Lysosomal Storage Diseases**

Lysosomal storage disorders (LSD) encompass 50 related conditions in which defective lysosomal hydrolysis results in an accumulation of toxic macromolecules (Vellodi, 2005). Upregulation of TFEB has been shown to increase clearance of these macromolecules in several models of LSD, namely, mucopolysaccharidosis, lipofuscinosis (Batten disease), and Pompe’s disease (Medina et al., 2015; Spamanato et al., 2013). In addition to the upregulation of lysosomal biogenesis, the benefit of TFEB upregulation in LSD has also been credited to its role in increasing exocytosis, a process by which a local spike in calcium stimulates fusion of lysosomes with the plasma membrane, allowing release of their degraded contents (Medina et al., 2015). In addition to increasing lysosome number, TFEB also regulates this stimulatory calcium flux through upregulation of the cation channel mucolipin 1 (MCOLN1) (Medina et al., 2015).

Given the benefit of increased TFEB activation in conformational diseases such as neurodegeneration and LSD, researchers have explored methods of inducing its translocation, namely through inhibition of mTORC1. Such inhibitors include rapamycin, rapalogs and ATP-competitive inhibitors (Pallet and Legendre, 2013). However, clinical use of these drugs has been
limited due to their unpredictability and adverse side effects profiles, especially in the kidney. Rapamycin analogs, (e.g sirolimus, everolimus and temsirolimus) for example, have been shown to induce proteinuria or worsen pre-existing proteinuria (Diekmann et al., 2012), a side effect that would preclude their use for the clearance of misfolded proteins in CKD, if found to be a pathogenic feature of the disease.

Given the adverse kidney risks with mTORC1 inhibitors, identifying mTORC1 independent methods of regulating TFEB nuclear translocation may allow for therapeutic upregulation of TFEB mediated pathways without adverse side effects on renal function. Interestingly, while modulation of TFEB’s phosphorylation status is the most widely studied form of its regulation, recent literature points to another form of post-translational regulation of TFEB, namely, through its acetylation (Bao et al., 2016). Therefore, just as kinases impart regulation of TFEB’s phosphorylation, regulators of acetylation may offer insight into novel mechanisms of regulating TFEB cellular localization and function. One enzyme that affects protein acetylation and controls nuclear translocation is histone deacetylase 6 (HDAC6).

4 HDAC6

4.1 Overview of Protein Acetylation

Post translational modification is the covalent modification of chemical moieties to a protein following its biosynthesis, commonly through an enzymatic reaction. In this way, post translational modification increases the diversity of protein behavior and enables cells to adapt to
changing internal and external microenvironments. The most studied form of post translational modification is (de)phosphorylation, the addition of a phosphate group regulated by kinase and phosphatase enzymes. As changes in post-translational phosphorylation have been implicated in disease states, the development of kinase inhibitors has enabled the in depth study of therapeutic regulators of phosphorylation in the treatment of disease (Li et al., 2013). The addition or removal of acetyl groups to proteins is a chemical modification known as (de)acetylation. These reactions are regulated by a family of enzymes known as histone deacetylases and histone acetyltransferases because they were first recognized for their role in (de)acetylating histones proteins (Brown et al., 2000).

Two major types of protein acetylation have been described. The first type is the transfer of an acetyl group to a nitrogen, a co-translational process occurring on the N-terminus of a growing peptide (Polevoda and Sherman, 2000). The second type of acetylation is lysine acetylation. This occurs on the ε-amino group of lysines on the N termini of histones and other proteins. Histone acetyltransferases (HATS) add acetyl groups at these sites. HATS decondense the surrounding chromatin and promote transcription. In contrast, histone deacetylases (HDACs) remove acetyl moieties, and on histones, this results in chromatin condensation and transcriptional repression (Brown et al., 2000).

Whereas HATS and HDACS were first appreciated for their role in (de)acetylation of histones, they also have numerous non-histone substrates throughout the cell. In fact, the number of
acetylated substrates, or the acetylome, rivals the phosphoproteome in size, with one study identifying 3600 acetylation sites on 1750 proteins (Choudhary et al., 2009).

4.2 The 18 Members of the Histone Deacetylase Family

The HDAC family consists of 18 isoforms that are categorized into four classes based on their homology to yeast deacetylases: class I (HDAC1, 2, 3 and 8), class II, subdivided into class IIa (HDAC4, 5, 7, 9) and IIb (HDAC6 and HDAC10), and class IV (HDAC11). These enzymes contain a zinc binding domain in their catalytic site. Broadspectrum HDAC inhibitors can chelate zinc at these sites and inhibit catalytic activity of HDAC enzymes. Class III HDACs are non-zinc dependent and instead, exert their catalytic function through an NAD^+ dependent mechanism. Whereas most members of the HDAC family function to modulate histone acetylation and transcription, a few members regulate cellular function through cytosolic substrates. HDAC6 is unique in its subcellular localization. Unlike most HDACs which reside in the nucleus, HDAC6 is a predominantly cytosolic protein. Because of this localization, HDAC6 may exert its enzymatic effects on a wide array of cytosolic substrates (Batchu et al., 2016).

4.3 The Cytosolic HDAC: Histone Deacetylase 6 (HDAC6)

Mammalian HDAC6 was discovered in 1990 based on its homology with the *Saccharomyces cerevisiae* histone deacetylase, HDAC1 (Grozinger et al., 1999; Verdel and Khochbin, 1999). In humans, HDAC6 is encoded on chromosome Xp11.22-23 (Voelter-Mahlknecht and Mahlknecht, 2003). An analysis of global expression patterns of HDAC6 showed that HDAC6 is most highly
expressed in the renal tubules of the kidney and seminiferous ducts of the testis (Uhlen et al., 2015). HDAC6 is 1215 amino acids in length and has a molecular weight of 131 kDa. Human HDAC6 contains an N-terminal nuclear export signal (Verdel et al., 2000) and a Ser-Glu tetrapeptide motif, both of which are responsible for the cytosolic localization of HDAC6. It also contains an N-terminus nuclear localization signal, which, when acetylated, sequesters HDAC6 in the nucleus and affects its catalytic function (Han et al., 2009; Liu et al., 2012b). Unlike other HDACs, HDAC6 contains a full duplication of its catalytic deacetylase domains (DD), termed DD1 and DD2. In addition, HDAC6 contains non-catalytic binding regions, namely, a dynein binding domain and a C-terminus ubiquitin binding zinc finger domain (ZnF-UBP or BUZ domain), allowing the protein to exert noncatalytic regulation of various cellular processes (Seigneurin-Berny et al., 2001). The structure of HDAC6 is shown in Figure B.

Figure B. Structure of HDAC6. The protein possesses two NES. Human HDAC6 also contains a SE14 motif that helps to retain the enzyme within the cytoplasm. A NLS at the N-terminal helps the protein to shuttle between the nucleus and the cytoplasm. There are two catalytic domains (DD1 and DD2). A dynein motor-binding domain and a ZnF-UBP are important for the non-enzymatic actions of the protein (Batchu et al., 2016).
4.4 HDAC6 Enzymatic Substrates

Over the past two decades, a number of HDAC6 cytosolic substrates have been identified. The microtubular protein α tubulin has been the most extensively studied with several independent reports showing that HDAC6 deacetylates α-tubulin on lysine residue 40 (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). By reducing the acetylation status of α-tubulin, HDAC6 has been implicated in microtubule stability and cytoskeletal dynamics (Valenzuela-Fernandez et al., 2008). Because α-tubulin is ubiquitously expressed, and deacetylated by HDAC6 across cell types, the hyperacetylation of α-tubulin is an established marker of HDAC6 inhibition or depletion (Zhang et al., 2003). This has served as an important marker in testing the efficacy of HDAC6 specific inhibitors which would be expected to increase α-tubulin acetylation levels. Additional substrates include the redox regulatory protein peroxiredoxin (Parmigiani et al., 2008), the cytoskeleton associated protein cortactin (Zhang et al., 2007) and the chaperone binding protein heat shock protein 90 (HSP90) (Kovacs et al., 2005).

4.5 Non-Enzymatic Actions of HDAC6

Beyond its catalytic DD1 and DD2 domains, HDAC6 exerts non-enzymatic effects on cellular function through its non-catalytic domains (Figure A). Through its ubiquitin binding zinc finger (Zn-UBP) domain, and its dynein binding domain, HDAC6 interacts with polyubiquitinated proteins and shuttles them as cargo through retrograde transport along microtubules, aggregating them into large, insoluble protein structures known as aggresomes (Kawaguchi et al., 2003b). These aggresomes are then tagged as substrates for clearance through the autophagy-lysosomal pathway (Kopito, 2000). Indeed, HDAC6 plays a key role in the cellular response to protein
misfolding through both its catalytic and non-catalytic functions as elaborated upon in section 5.2 below.

5 HDAC6 as a Potential Regulator of TFEB

5.1 HDAC6 in Nuclear Translocation of Transcription Factors

As a cytosolic deacetylase, HDAC6 has been shown to regulate the nuclear shuttling of multiple transcription factors through post-translational modification of lysine acetylation. This makes it a plausible candidate in the search for deacetylase-mediated regulation of transcription factor EB nuclear translocation. There are several examples by which HDAC6 regulates transcription factor shuttling. Under basal conditions, HDAC6 forms a tri-complex with the chaperone protein HSP90 and the transcription factor heat-shock transcription factor 1 (HSF1) (Boyault et al., 2006b), sequestering HSF1 in the cytosol. Upon sensing cellular stress as evidenced by an increase in misfolded proteins, HDAC6 dissociates from the complex, leading to the nuclear translocation of HSF1 and subsequent transcription of molecular chaperones for protein folding (Boyault et al., 2006b). Similarly, HDAC6 imparts regulation of glucocorticoid receptor nuclear translocation through a HSP90 dependent mechanism (Kovacs et al., 2005). Because of this role, HDAC6 inhibitors have shown promise in increasing nuclear translocation and, subsequent upregulation of downstream pathways. For example, HDAC6 mediated deacetylation of the protein survivin leads to its cytoplasmic retention (Riolo et al., 2012), and inhibition of HDAC6 consequently increases nuclear translocation of survivin in breast cancer cells (Lee et al., 2016). Other such transcriptional regulators subject to HDAC6 post translational modification include: runt-related transcription factor 2 (RUNX2) (Westendorf et al., 2002), nuclear factor kappa-light-chain
enhancer of activated B cells (NF-κB) (Zhang and Kone, 2002) and the nuclear receptor corepressor ligand-dependent corepressor (LcoR) (Palijan et al., 2009). In our search to identify novel deacetylase regulators of transcription factor EB, HDAC6 satisfied our first query surrounding a deacetylase enzyme that is known to regulate nuclear translocation of transcription factors and misfolded protein disposal.

5.2 HDAC6 in Misfolded Protein Clearance

In addition to its role in transcription factor shuttling, HDAC6 has also been shown to play multiple roles in the regulation of autophagy. The unique structure, and cytosolic localization of HDAC6 lends itself to this function. The ZnF-UBP domain at its C-terminus enables HDAC6 to bind to ubiquitinated proteins and the dynein motor binding domain enables HDAC6 to bind to dynein. Dynein is a motor protein that uses ATP to migrate along microtubules generally through retrograde transport towards the nucleus. Thus, the binding of HDAC6 to dynein enables the transport of its cellular cargo of misfolded proteins along microtubules into a growing protein aggregate structure known as the aggresome (Johnston et al., 2002) as shown in Figure C. As such, HDAC6 is considered to favour the accumulation of misfolded proteins into aggresomes and decreases their clearance through the UPS by reducing the catalytic activity of the 26S proteasome (Boyault et al., 2006a). Interestingly, although the physical interactions between ubiquitinated proteins, HDAC6 and dynein motors are mediated by its non-catalytic ubiquitin binding domain, deacetylase activity is required for this function, with the reintroduction of deacetylase-deficient HDAC6 to HDAC6 knockout cells being unable to restore aggresome formation (Kawaguchi et al., 2003a). Further down the pathway of misfolded protein degradation, HDAC6 also functions to recruit and deacetylate cortactin, which is necessary for
autophagosome-lysosome fusion under basal conditions (Lee and Yao, 2010) as seen in Figure C. Despite these apparently enabling actions of HDAC6, its role appears far more complex under disease settings, in which HDAC6 appears dispensable in promoting autophagosome-lysosome fusion (Lee et al., 2010) and in some cases, HDAC6 inhibition actually promotes protein clearance (Selenica et al., 2014) in neurodegenerative disease, a disease characterized by the accumulation of misfolded proteins. Secondly, misfolded protein accumulation is a feature of certain cancer therapies that exert their cytotoxic effects through inhibition of the proteasome and it is in these two major disease classes that the effects of HDAC6 inhibitors have been most extensively investigated to date.
HDAC6 binds ubiquitinated proteins through its ZnF-UBP domain and, after binding to dynein, transports its misfolded cargo along microtubules towards perinuclear aggresomes. Aggresomes are disposed of by autophagy and HDAC6 itself facilitates autophagy completion by recruiting and deacetylating cortactin, which is necessary for fusion of autophagosomes with lysosomes. HDAC6 also forms a tri-complex with HSP90 and HSF1. On sensing of ubiquitinated aggregates, HDAC6 dissociates from this tri-complex, allowing HSF1 migration to the nucleus and the transcription of molecular chaperone HSPs (Batchu et al., 2016).

6 Pharmacological Inhibitors of HDAC6

There are three broad categories of HDAC inhibitors: “pan” or broad-spectrum inhibitors, class-specific inhibitors, and isoform-specific inhibitors. Two pan-HDAC inhibitors that have reached the clinic, vorinostat (also known as suberoylanilide hydroxamic acid, SAHA) and romidepsin, both inhibit zinc-dependent HDAC isoforms. Structurally, these HDAC inhibitors are composed of a zinc binding group, namely, hydroxamic acid, thiol, carboxylic acid, ketone or substituted aniline, that chelates zinc ions at the catalytic site; a linker domain and a cap group that blocks
binding of the substrate to the binding pocket (Dallavalle et al., 2012; Li et al., 2013) as shown in Figure D. Variations in the cap region can confer isoform specificity because HDAC enzymes differ in the pockets surrounding their enzymatic binding region (Nielsen et al., 2005). Whereas these pan-HDAC inhibitors have gained regulatory approval for the treatment of some hematological malignancies (Hymes, 2010), their use for the treatment of chronic conditions has been limited by their hematological toxicity and QT prolongation (Shultz et al., 2011).

![Figure D](image)

**Figure D.** Typical structure of HDAC inhibitors. Most HDAC inhibitors are made up of a zinc-binding group which chelates the zinc ion at the enzyme’s active site joined by a linker region to a cap group which binds to the substrate-binding region of the enzyme. The figure shows the HDAC inhibitor structure as it would fit within the catalytic DD2 region of HDAC6 (Batchu et al., 2016).

Unlike the deletion of other HDACs, deletion of HDAC6 yields a comparatively benign phenotype in mice, suggesting that inhibiting this particular isoform may be better tolerated. Specifically, whereas the genetic deletion of a number of HDAC isoforms (Haberland et al., 2009; Lagger et al., 2002; Montgomery et al., 2007; Montgomery et al., 2008; Vega et al., 2004) has led to perinatal lethality, HDAC6 knockout mice are viable and develop normally with only minor abnormalities in cancellous bone density and a mildly underdeveloped immune response (Zhang et al., 2008).
Tubacin, which stands for tubulin acetylation inducer (Haggarty et al., 2003b), was the first generation of HDAC6 specific inhibitors. Identified from a screen of 7392 small molecule inhibitors, it consists of a large cap composed of six hydrophobic rings and a 1,2 dioxane ring. Its success as an HDAC6 specific inhibitor was evidenced by a marked increase in α-tubulin acetylation, without altering histone acetylation. However, the application of tubacin for in-vivo use has been limited due to its inefficient biosynthesis, hydrophobicity and lack of drug like structure (Haggarty et al., 2003a; Haggarty et al., 2003b).

The HDAC6 inhibitor that has been most widely reported on in the biomedical literature to date is Tubastatin A, the synthesis of which was originally described by Butler and co-workers in 2010 (Butler et al., 2010a; Butler et al., 2010b). The rational design of Tubastatin A is especially interesting. To select for isoform specificity, the investigators set out to compare HDAC6 with the Class I HDAC isoform, HDAC1. Because crystal structures have not been defined for HDAC6 and HDAC1, the investigators instead elected to use a bioinformatic tool for predicting protein structure based upon amino acid sequence (Roy et al., 2010). By comparing the modeled catalytic pockets of HDAC1 and HDAC6, they discovered that although the active site is conserved, the catalytic channel rim differs between the two isoforms being substantially wider in HDAC6 than HDAC1 (Butler et al., 2010b). The investigators therefore set out to design compounds based upon the canonical HDAC inhibitor structure (i.e. zinc binding group [hydroxamic acid], linker and cap group) with a cap group that was large enough and inflexible enough to occupy the catalytic channel rim of HDAC6 but not HDAC1 (Butler et al., 2010b). The cap group that best fulfilled these requirements was the tricyclic structure of a carbazole cap.
(Butler et al., 2010b). However, carbazoles are generally too lipophilic to make good drugs offering suboptimal ADMET (absorption, distribution, metabolism, excretion and toxicity) properties (Arnott and Planey, 2012). So, the investigators introduced a tertiary amine to disrupt the planarity of the tricyclic ring and reduce lipophilicity (Butler et al., 2010b). Finally, recognizing that the modeled catalytic channels of HDAC1 and HDAC6 also differ, with the HDAC6 channel being wider and shallower, the investigators sought to adapt the linker region, replacing the typical alkyl chain with bulkier and shorter aromatic moieties (Butler et al., 2010b). The result was the synthesis of Tubastatin A, which has an IC₅₀ for HDAC6 of 0.015 µM, representing >1000-fold selectivity versus all other HDAC isoforms (except HDAC8, 57-fold selectivity) (Butler et al., 2010b). In primary cultured neurons, Tubastatin A increased α-tubulin acetylation without affecting histone acetylation and it dose-dependently protected against oxidative stress-induced neuronal death (Butler et al., 2010b).

Whereas Tubastatin A has been relatively widely adopted into pre-clinical mechanistic studies, the only preferentially HDAC6-specific inhibitor to have reached clinical trial is rocilinostat. Rocilinostat is a hydroxamic acid derivative with an IC₅₀ for HDAC6 of 5nM. However, it also has activity against other HDAC isoforms with IC₅₀s for HDACs 1, 2, 3 and 8 of 58 nM, 48 nM, 51 nM and 100 nM respectively (IC₅₀ >1 µM for the other HDAC isoforms) (Santo et al., 2012). As with other HDAC6 inhibitors, rocilinostat dose-dependently increased α-tubulin acetylation without affecting the acetylation status of histone proteins (Santo et al., 2012). It also induced less cytotoxicity in peripheral blood mononuclear cells and T cells than the pan-HDAC inhibitor, vorinostat (Santo et al., 2012). Rocilinostat has mostly been studied for its role in combination
with proteasome inhibitors for the treatment of multiple myeloma or lymphoid malignancies (Amengual et al., 2015; Dasmahapatra et al., 2014; Mishima et al., 2015; Santo et al., 2012).

Although Tubacin, Tubastatin A and rocilinostat have been the most extensively studied agents to date, other HDAC6 inhibitors have also been synthesized. In 2008, Kozikowski and co-workers reported the synthesis of HDAC inhibitors containing a phenylisoxazole as the cap group, generating an HDAC6 inhibitor with picomolar potency (Kozikowski et al., 2008). Arylalanine containing hydroxamic acids have also been reported as another class of HDAC6 selective inhibitors, potent in low micromolar concentrations (Schafer et al., 2008; Schafer et al., 2009). Because most HDAC inhibitors share a common structure, to enhance the HDAC inhibitor pool, Inks and co-workers elected to screen the Library of Pharmacologically Active Compounds for agents that exhibit HDAC inhibitory properties in a search for novel compounds with a novel structure (Inks et al., 2012). Out of the library of 1280 compounds, they identified five with HDAC inhibitory properties, one of which (a dual-specificity phosphatase inhibitor, NSC-95397) being selective for HDAC6 (Inks et al., 2012). A number of analogues of the parent compound were synthesized and one, NQN-1, demonstrated an IC$_{50}$ for HDAC6 of 5.5 µM, with minimal inhibitory activity against other HDAC isoforms (Inks et al., 2012). Molecules with a cyclic peptide scaffold or chiral structure derivatives (Olsen and Ghadiri, 2009; Smil et al., 2009) and sulfamide- (Jones et al., 2006), thiolate- (Itoh et al., 2007), trithiocarbonate- (Dehmel et al., 2008) and mercaptoacetamide- (Kozikowski et al., 2007) based compounds have also been explored as potential selective HDAC6 inhibitors.
Like TFEB, the implication of HDAC6 in maintaining proteostasis highlights it as a potential therapeutic target. Indeed, HDAC6 inhibitors have found preliminary success in multiple disorders involving misfolded protein accumulation. Interestingly however, the collective insights indicate that the role of HDAC6 is likely to be more complicated than simply being protective or detrimental, and is likely related to the multifaceted role of HDAC6’s catalytic and non-catalytic actions in the autophagy pathway.

*Neurodegeneration*

In the case of Parkinson’s disease, HDAC6 promotes aggregate formation and protects dopaminergic neurons from the injurious cellular effects of α-synuclein (Du et al., 2010) and, in brain sections from people with Parkinson’s disease, Lewy bodies are enriched for HDAC6 (Kawaguchi et al., 2003a). Together, these observations suggest that HDAC6 upregulation in brain tissue of people with Parkinson’s disease may be a protective response suggesting that therapeutic augmentation of HDAC6 may slow the progression of the disease (Yan, 2014).

In contrast however, the role of HDAC6 in the context of tauopathies and Alzheimer’s disease is less clear. Tau is a client protein for HSP90 (Karagoz et al., 2014) and HDAC6 levels correlate with tau burden, with a decrease in HDAC6 expression or activity favouring clearance of tau, potentially through the promotion of HSP90 acetylation and consequent attenuation of its tau-chaperoning actions (Cook et al., 2012). Even though HDAC6 has been associated with
Alzheimer’s disease in a number of studies, its precise role has not yet been fully established. Early upregulation of HDAC6 may confer protective benefits, but overtime this may lead to accelerated neuronal damage (Zhang et al., 2013). Nonetheless, two separate groups have each recently reported an improvement in cognition with HDAC6 inhibition in mouse models of Alzheimer’s disease (Selenica et al., 2014; Zhang et al., 2014).

*Cancer*

Whereas HDAC6 undoubtedly plays a role (albeit complex) in the pathogenesis of or protection against neurodegenerative disease, to date clinical trials of HDAC6 inhibitors have been restricted to the treatment of certain malignancies. The link between HDAC6 and aggresome formation represents probably the most clearly defined and (at present) clinically significant relationship between modulation of HDAC6 activity and altered cancer outcomes. Transformed cells accumulate misfolded proteins at a faster rate than non-transformed cells and, for cancer cell survival, these misfolded proteins must be appropriately disposed of through either the UPS or the aggresome-autophagy pathway (Rodriguez-Gonzalez et al., 2008). Proteasome inhibitors prevent disposal of misfolded proteins by the UPS and their use in combination with HDAC6 inhibitors may promote cytotoxicity by inhibiting both the UPS and the aggresome-autophagy pathway (Hideshima et al., 2005). However, although HDAC6 inhibition may promote cell death in cancer, it may serve a protective role in non-cancer cells, as has been noted in chronic conditions such as cardiovascular and renal diseases.
Cardiovascular disease

Cardiomyocytes are essentially post-mitotic and therefore unable to regenerate. As a result, they are vulnerable to the deleterious effects of the accumulation of misfolded proteins, which can cause heart failure. McLendon and co-workers observed that hyperacetylation of α-tubulin occurred in a mouse model of proteinopathy-induced heart failure (McLendon et al., 2014). Reasoning that this is an adaptive response, the investigators observed that knockdown or inhibition of HDAC6 increased autophagy and reduced aggresome accumulation in cultured cardiomyocytes and that pan-HDAC inhibition in-vivo prevented aggresome formation and improved cardiac function (McLendon et al., 2014). Because the aging heart has a reduced capacity to remove protein aggregates (De Meyer et al., 2010), this has led investigators to postulate that HDAC6 inhibition may improve cardiac function in the elderly given the relationship between aging and impaired autophagy (Ferguson and McKinsey, 2015).

8 HDAC6 and Kidney Disease

Whereas the contribution of HDAC6 to the regulation of misfolded protein clearance in CKD remains the topic of this thesis, it is worth noting that preliminary research suggests that inhibition of HDAC6 may be protective in the kidney generally. This is interesting, given that the kidney is one of the sites where HDAC6 is most highly expressed. In terms of pathology, HDAC6 may play a role in renal fibrosis as evidenced by a requirement for HDAC6 in transforming growth factor-β (TGF-β) induced epithelial to mesenchymal transition (Shan et al., 2008) and a reduction in TGF-β expression in the kidneys of angiotensin II-infused mice treated with Tubastatin A (Choi et al., 2015a). Separately, HDAC6 has also been implicated in cystic diseases of both the liver
(Gradilone et al., 2014) and the kidney (Mergen et al., 2013). This association likely relates to the importance of HDAC6 in the formation of the primary cilium. Nearly all mammalian cells possess a single primary cilium. Far from being vestigial organelles, primary cilia play an important role in intracellular signaling and in the regulation of cell division through their assembly and disassembly, their dysfunction contributing to renal diseases such as polycystic kidney disease (Singla and Reiter, 2006). Because cyst growth occurs as a result of persistent proliferation of de-differentiated epithelial cells (Wilson, 2004), dysregulation of HDAC6 can impair ciliary disassembly and contribute to the development of renal cysts due to impaired cell division regulation (Mergen et al., 2013).

The regulation of primary cilium disassembly is not the sole mechanism through which HDAC6 may contribute to the development of renal cysts. Through its α-tubulin deacetylating actions, HDAC6 also regulates the intracellular transport of the epidermal growth factor receptor (EGFR) (Gao et al., 2010), whose increased activity promotes cyst formation (Richards et al., 1998). In kidney epithelial cells with a mutation in the \textit{PKD1} gene, that encodes the protein polycystin-1 and that is associated with autosomal dominant polycystic kidney disease, HDAC6 expression was observed to be increased, whereas HDAC6 inhibition promoted EGFR degradation and normalized EGFR localization (Liu et al., 2012a). Autosomal dominant polycystic kidney disease can be caused by mutations in either the \textit{PKD1} gene or in the \textit{PKD2} gene, the latter encoding the protein polycystin-2. Polycystin-1 and -2 interact with each other (Cebotaru et al., 2014). Separate to its role in EGFR trafficking, HDAC6 also binds polycystin-2 and expression of full-length polycystin-1 accelerates transport of the polycystin-2/HDAC6 complex towards aggresomes, facilitating the degradation of polycystin-2 by autophagy and thus negatively
regulating its expression (Cebotaru et al., 2014). The balance between increased and decreased activity of polycystin-1 and -2 therefore appears to be tightly regulated in renal epithelial cells and either upregulation or downregulation of either protein may result in cyst formation (Cebotaru et al., 2014). It is possible that inhibiting HDAC6 can redress an imbalance in polycystin-1/2 activity attenuating the development of renal cysts. Indeed, Cebotaru and colleagues recently showed that pharmacological inhibition of HDAC6 with Tubacin slowed renal cyst growth and improved kidney function in a rodent model of polycystic kidney disease (Cebotaru et al., 2016).

In summary, despite its name, HDAC6 is unique from other HDAC isoforms in its cytoplasmic functionality and in its druggability. It deacetylates non-histone proteins and, independent of its catalytic activity, it acts as a bridge linking the UPS and the aggresome-autophagy pathway, regulating the disposal of misfolded proteins. It also plays an important role in transcription factor nuclear translocation and therefore, can impart regulation on transcriptional networks. HDAC6 expression or activity is altered in cancer, neurodegenerative diseases, cardiovascular disease and other diseases, where it may contribute to the pathogenesis of the condition or play a compensatory role (Figure E). In the kidney, HDAC6 inhibition may serve a protective role, but whether this protection is related to its autophagic activity remains to be seen. While knowledge about TFEB in the kidney is limited, its success in clearing misfolded proteins in other disease settings highlights its role as a potential therapeutic target. Since current therapies aimed at mediating its phosphorylation status are limited due to renal toxicity, modifying the acetylation status of TFEB may offer another avenue of regulation. Therefore, we set out to determine if and to what extent misfolded proteins accumulate in CKD; whether misfolded protein accumulation
is linked to TFEB; and whether HDAC6 is involved and can itself alter TFEB activity in kidney cells.

Figure E. Conditions associated with altered HDAC6 activity or in which HDAC6 inhibition may confer therapeutic benefit. HDAC6 inhibition has been most extensively studied for its role in the treatment of haematological malignancies and HDAC6 itself has been implicated in the pathogenesis (or protection against) a number of neurodegenerative diseases. The protein may also play important roles in other forms of cancer, in cardiovascular disease and in inflammation, whereas its actions in the development of mood disorders and kidney diseases and in the regulation of thrombosis and haemostasis are beginning to be recognized (Batchu et al., 2016).
Chapter 2

Hypothesis and Research Aims

1 Hypothesis

There is growing appreciation for the sensitivity of proximal tubule cells to impaired proteostasis with recent studies pointing to chronically impaired quality control mechanisms as a precursor to tubule cell apoptosis and a decline in renal function. Although the contribution of the autophagy-lysosomal pathway has been studied in conformational disorders, its contribution to renal disease is less clear. Transcription factor EB (TFEB) has been described as a master regulator of the autophagy-lysosomal pathway and we hypothesize that kidney disease may be associated with 1) dysregulation of renal TFEB and 2) may manifest as an accumulation of misfolded proteins. Current therapies aimed at increasing TFEB activation are limited due to adverse renal outcomes. Recent research has uncovered a role for TFEB acetylation as an alternative method of regulation. HDAC6 is a known regulator of transcription factor shuttling and its inhibition has improved protein clearance in conformational diseases. Therefore, we hypothesize that histone deacetylase (HDAC6) may impart a regulatory role on TFEB activation and that inhibition of HDAC6 may activate TFEB mediated autophagy-lysosomal pathways, improve cellular misfolded protein clearance and serve a renoprotective role.
2 Research Aims

1) To determine whether TFEB expression levels are dysregulated in kidneys from humans with diabetic kidney disease and rats with CKD induced by subtotal nephrectomy surgery.

2) To assess the degree of misfolded protein aggregates in kidneys from humans with diabetic kidney disease and in kidneys from subtotally nephrectomized rats.

3) To determine whether HDAC6 inhibition alters TFEB activity.

4) To determine whether HDAC6 inhibition attenuates CKD progression in subtotally nephrectomized rats and whether this is associated with altered TFEB activity and misfolded protein accumulation.

The remainder of this thesis details materials and methodology used to assess these aims, results, conclusions, discussion on the implications of these findings, limitations of the current study and suggestions for future research.
Chapter 3

Materials and Methods

1  Human Studies

Archival formalin-fixed, paraffin-embedded kidney tissue was examined from 12 patients with diabetic glomerulosclerosis and 12 individuals without diabetes. The study was approved by the Nova Scotia Health Authority Research Ethics Board and the Research Ethics Board of St. Michael’s Hospital and was conducted in accordance with the Declaration of Helsinki.

2  Real-Time PCR

2.1  RNA Isolation

RNA was extracted from cultured cells using Trizol reagent (ThermoFisher Scientific, Waltham, MA). Briefly, samples were incubated with Trizol for 5 minutes before the addition of 200 µL of chloroform. Following five seconds of rapid agitation, samples were centrifuged at 12,000 g for 15 minutes to allow for phase separation. RNA was precipitated from the aqueous phase by addition of 500 µL of 100% isopropanol. Following incubation and centrifugation at 12,000 g for 10 minutes, supernatants were removed and RNA pellets were washed with 75% ethanol, air-dried, re-suspended in RNAse DNase free water and heated at 60°C prior to quantification.
RNA was extracted from paraffin embedded human kidney sections using the Qiagen RNeasy FFPE Kit and was extracted from rat kidney tissue using the Qiagen RNeasy Mini Kit as per the manufacturer’s instructions (Qiagen, Hilden, Germany). RNA concentration was determined by light absorbance of RNA and DNA at wavelengths of 260 nm and 280 nm on a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific), with a 260/280 nm ratio of greater than 1.8 used for cDNA synthesis. Similarly, RNA integrity was further assessed using the Agilent 2100 Bioanalyzer using the RNA 6000 NanoChip kit for total eukaryotic RNA (Agilent Technologies, Santa Clara, CA). Samples with an RNA integrity number (RIN) of greater than 7 were used for cDNA synthesis.

### 2.2 First Strand cDNA Synthesis

RNA (1 µg total RNA) was reversed transcribed to 20 µL reaction volume using 1 µL of oligo(dT)$_{20}$, 1 µL of 10 mM dNTP mix, sterile distilled water, 4 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT, 1 µL of 200 units/µL SuperScript III Reverse Transcriptase with the recommended thermocycler settings. All reagents for cDNA synthesis were purchased from ThermoFisher Scientific.

### 2.3 Primer Selection

Primer sequences were designed using the online primer design tool Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Gene transcript FASTA sequences were obtained from NCBI’s Nucleotide database and entered into Primer Blast. Selected primer sequences were double-checked in Primer Blast to ensure transcript specificity. Primers were
purchased from Integrated DNA Technologies (IDT) (Coralville, IA) and these sequences were
as follows: human TFEB, forward GTAGAGATGATGCCTCGCA, reverse CAGCCTGAGCTTGCTGTCAT;
human RPL32, forward CAACATTGGTTATGGAAGCAACA, reverse TGCAGCTTGCGACCAGGAAC;
rat TFEB, forward AGATCTGCTTCTTCTTGCGA, reverse GCAGCAAACCTGTTGCGTA; rat
RPL13a, forward ATGAACACCAACCCGCGTC, reverse GCCTCTTTTGGTCTTGTGCG; rat
LAMP1, forward AGAAGGCTCCACGCAATTGA, reverse TGCAGCCTAACCACCACAG.

2.4 Plate Preparation and Analysis

Gene expression was assessed using SYBR green master mix (Wisent, Saint-Jean-Baptiste, QC)
on a ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific). Samples were loaded onto a
386 well block, set at standard thermocycler conditions and relative gene expression was assessed
using the Comparative $C_T$ method. $C_T$, or threshold cycle, is the PCR cycle at which the
fluorescent signal of the reporter dye crosses a set threshold. The relative gene expression of the
gene of interest was expressed as a fold change relative to an internal control (housekeeping) gene
that displays stable expression levels across treatment groups. The fold change was calculated
from the following equation (Schmittgen and Livak, 2008):

$$\text{Fold change} = 2^{\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample B})]$$
3 Immunoblotting

3.1 Lysate Preparation

NRK-52E Cells

NRK-52E cells were cultured in 6 well plates. Following treatment, cells were washed twice with sterile PBS and then collected by scraping wells with 70-100 μL of homogenization buffer with phosphatase inhibitor (Appendix). Cells were homogenized using a hand held homogenizer prior to protein quantification.

Tissue

Tissues were dissected with cleaned tools, placed in Eppendorf tubes and snap frozen in liquid nitrogen prior to storage at -80 °C. During homogenization, 300 μL of cold homogenization buffer with phosphatase inhibitor (Appendix) were added per 5 mg of tissue. Samples were homogenized with an ultrasonic homogenizer, model 3000 (BioLogics Inc., Manassas, VA) prior to protein quantification.

Nuclear fractionation

Nuclear fractionation was performed by centrifuging samples for 1 minute at 16,000 x g at 4°C and collecting the supernatant for use as the cytosolic fraction. The resulting pellet was resuspended in homogenization buffer and subsequently centrifuged for 15 minutes at 16,000 x g at 4°C. The supernatant was isolated for use as the nuclear fraction. Equal amounts of cytosolic and nuclear protein were assessed by Western blot.
3.2 Protein Concentration Determination

Protein concentration was determined using Bio-Rad’s Quick Start Bradford 1X Dye Reagent (Bio-Rad, Hercules, CA) which assesses Coomassie Brilliant Blue G-250 dye binding to proteins. The standard of Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO) was used in serial dilutions ranging from 2 mg/mL to 0 mg/mL. Standards and samples (5 µL) were pipetted into a 96 well plate in duplicate. Then, 245 µL of dye reagent was added to each well and incubated at room temperature for 5 minutes. Samples were assessed on a SpectraMax M5e spectrophotometer (Molecular Devices, Sunnyvale, CA) set at an excitation wavelength of 595 nm.

3.3 Gel Electrophoresis

Lysed proteins were denatured by addition of 5 µL of 5X lane marker reducing sample buffer (ThermoFisher Scientific), and boiled in a heat block set at 100°C for 5 minutes. Following boiling, 25 µg of total protein was loaded into wells of 10% SDS-PAGE gels, along with 5 µL of Precision Plus Protein Dual colour standard (Bio-Rad) as the molecular weight protein ladder. The gel was placed in a Mini-Protean Tetra System (Bio-Rad), the tank was filled with 1L of 1X running buffer (Appendix) and ran at 100V for 1-2 hours at room temperature.

3.4 Membrane Transfer

Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Two filter papers, two sponges and one nitrocellulose membrane were soaked in 1X transfer buffer containing 20% methanol (v/v) per gel (Appendix). Gels were removed from glass plates and loaded into the transfer
cassette in the following order: sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge. Transfer was run at 100V for two hours at 4°C.

3.5 Blocking the Membrane and Antibody Incubation

Following transfer, nitrocellulose membranes were removed and submerged in 5% blocking solution (Appendix) for 1 hour, with constant agitation. After blocking, membranes were washed in fresh 1X TBS-T (Appendix) for 10 minute intervals, over a period of 30 minutes. Membranes were incubated with the recommended dilution of primary antibody prepared in 10 mL of 5% BSA in PBS, overnight at 4°C with constant agitation. Primary antibodies were prepared in the following concentrations: p62 1:1000 (Cell Signaling Technology), ubiquitin 1:1000 (Cell Signaling Technology), phospho-eIF2α 1:1000 (Cell Signaling Technology), eIF2α 1:1000 (Cell Signaling Technology), acetylated α-tubulin 1:1000 (Sigma-Aldrich), total α-tubulin 1:1000 (Sigma-Aldrich), cleaved caspase 3 1:1000 (Cell Signaling Technology, Danver, MA), TFEB 1:700 (Abcam, Cambridge MA), β-actin 1:10,000 (Sigma-Aldrich), acetylated lysine 1:1000 (Cell Signaling Technology) and histone H3 1:2000 (Cell Signaling Technology). Following primary incubation, membranes were washed in fresh TBS-T for 10 minute intervals over a 30 minute period. Then, they were incubated in the recommended dilution of horseradish peroxidase (HRP) conjugated secondary antibody (Bio-Rad) in 5% blocking solution for 1-2 hours. Membranes were then washed with TBS-T for 30 minutes prior to detection.
3.6 Detection and Analysis of Labelled Proteins

Proteins were detected using a luminol based enhanced chemiluminescent (ECL) substrate that reacts with HRP tagged proteins (ThermoFisher Scientific). Membranes were submerged in ECL for 4 minutes, with constant pipetting. Following incubation, membranes were placed between two transparent films in a Western blot cassette. In the dark room, film was placed over the membrane for 1-5 minutes, and repeated as necessary for optimal exposure prior to development. Alternatively, following ECL incubation, blots were exposed and photographed on a ChemiDoc Touch Imaging System (Bio-Rad) for 30 seconds-10 minutes as needed. Protein expression was quantified using densitometry measures on ImageJ 1.46r software (National Institute of Health, Bethesda, MD).

3.7 Re-probing the Nitrocellulose Membrane

Membranes were submerged in 1X Antibody Stripping Buffer (GeneBio-Application L.T.D, Kfar-HaNagid, Israel) for 15-30 minutes as needed. Membranes were washed with distilled water for 10 minutes prior to blocking and re-probing as stated above.

4 Cell Culture

4.1 Cell Lines Used

In-vitro experiments were conducted in proximal tubule lineage NRK-52E cells (ATCC, Manassas, VA) (Advani et al., 2009). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich), with 20% fetal bovine serum (FBS) or 10% FBS (Sigma-
Aldrich) when subjected to starvation conditions. Cells were incubated at 37°C and 95% O\textsubscript{2} and 5% CO\textsubscript{2}. Each experiment was performed in triplicate, with the exception of cells used for RTqPCR, which was performed with six replicates.

4.2 Tubastatin A Experiments

The efficacy of Tubastatin A in inhibiting HDAC6 in-vitro was determined by assessing for a dose dependent increase in \(\alpha\)-tubulin acetylation in NRK-52E cells. Cells were treated with vehicle (0.1% DMSO) or Tubastatin A (MedChem Express, Monmouth Junction, NJ) in the following concentrations: 0.6 \(\mu\)M, 1.2 \(\mu\)M, 2.5 \(\mu\)M, 5 \(\mu\)M, 10 \(\mu\)M. Cells were incubated with Tubastatin A for 24 hours before preparation of lysates for immunoblotting as discussed.

4.3 Endoplasmic Reticulum Stress Induced Apoptosis Experiments

Apoptosis was assessed in-vitro following treatment with the ER stress inducer, thapsigargin (Olsowski and Urano, 2011). NRK-52E cells were plated and serum starved overnight before pre-treatment with 2.5 \(\mu\)M of Tubastatin A or vehicle (0.1% DMSO). Following a 4 hour incubation period, the culture media was supplemented with 500 nM thapsigargin (Sigma-Aldrich) for 24 hours. Cell lysates were either collected for immunoblotting for cleaved caspase-3 (Cell Signaling Technology) or prepared for flow cytometric analysis of PE Annexin V labelling.
4.4 PE/Annexin V Apoptosis Detection Assay

Flow cytometric analysis of apoptotic cell death was determined using a PE Annexin V apoptosis detection assay (BD Biosciences, San Jose, CA). Following treatment, cells were washed twice with cold PBS and re-suspended in 1X binding buffer at a concentration of $1 \times 10^6$ cells/mL as per the manufacturer’s instructions. An aliquot of 100 µL of cells was supplemented with 5 µL of PE Annexin V and 5 µL of 7-Amino-actinomycin (7-AAD). Annexin V is a calcium-dependent phospholipid binding protein that binds to exposed phospholipid phosphatidylserine (PS) on the surface of apoptotic cells. Likewise, 7-AAD is a viability marker that enters cells when cell membranes are compromised, as is the case during late stage apoptosis and necrosis. Cells were incubated for 15 minutes, at room temperature in the dark, before the addition of binding buffer. Ten thousand events were analyzed for PE/Annexin V and 7AAD by flow cytometry using a MACSQuant (Miltenyi Biotec, Auburn, CA).

4.5 Immunoprecipitation

An immunoprecipitation experiment for TFEB was conducted in NRK-52E cells following treatment with Tubastatin A. Cells were plated in Petri dishes at approximately 70% confluency and treated with vehicle (0.1% DMSO) or 2.5 µM Tubastatin A for 24 hours. Cells were collected in 300 µL of homogenizing buffer and protein concentration was measured as described. Primary antibodies against TFEB (Abcam, Cambridge MA) were incubated with cell lysates at a ratio of 1 µg primary antibody to 500 µg of total protein. Negative controls were incubated with an equivalent ratio of goat IgG primary antibody (Abcam). Samples were incubated overnight, at 4°C under constant rotation conditions. The following day, agarose G beads (Roche, Basel, Switzerland) were prepared by washing beads in 1mL of chilled PBS, followed by centrifugation
at 1000 rpm for two minutes, three times. Beads were then added to each sample to incubate overnight, at 4°C under constant rotation. Afterwards, samples were centrifuged at 1000 rpm for two minutes. Supernatants were removed and the protein-bead mix was washed with 1 mL of chilled PBS and centrifuged (1000 rpm for 2 minutes) for eight consecutive cycles. Next, 5X lane marker reducing sample buffer (ThermoFisher Scientific) was added to each sample in a 1:5 μL ratio, and boiled for 10 minutes. An aliquot of 30 μL was obtained from the supernatant of each sample, and subjected to gel electrophoresis for immunoblot as described.

5 Animals

5.1 Subtotal Nephrectomy and Sham Surgery

The subtotally nephrectomized (SNx) rat model is generated by the complete removal of one kidney, and selective infarction of 2/3 of the remaining kidney, leading to progressive proteinuria and renal failure. Anesthesia was induced through inhalation of 2.5% isoflurane in a designated gas chamber. Pre-operative pain management was achieved by injection of 0.05 mg/kg buprenorphine by designated staff. Toe pinch reflex, body temperature and limb extremities were monitored to ensure the rats were anesthetized. Following confirmation, the abdomen of the rat was shaved and cleaned with alcohol and betadine. Using sterile technique, a 2-3cm incision was made through the skin and underlying muscle, exposing the right kidney. The kidney was resected, and the remaining kidney was ligated with a 4-0 silk suture. The peritoneum and abdominal muscle was closed with a 3-0 Vicryl suture and the overlaying skin was stapled. Sham surgery paralleled SNx surgery in methodology. Instead of removal, kidneys were manipulated prior to abdominal closure.
5.2 In-vivo Pharmacological HDAC6 Inhibition in Sham and SNx Rats

5.2.1 Administration of Tubastatin A In-vivo

Male Sprague Dawley rats were given subcutaneous injections of 30mg/kg Tubastatin A or vehicle (5% dextrose), three times a week for 3 weeks. Kidneys were collected and immunoblotted for acetylated α-tubulin. Following confirmation of HDAC6 inhibition with Tubastatin A, we proceeded to the interventional study. Male Sprague Dawley rats were subjected to SNx or Sham surgery as previously described. Then, urine protein excretion was assessed four weeks post-surgery to randomize rats to receive thrice weekly injections of vehicle or 30 mg/kg Tubastatin A for the remaining three weeks of the study. At week seven, urine protein excretion, systolic blood pressure and glomerular filtration rate were assessed. Following sacrifice, renal tissue was collected for structural and molecular biological analysis.

5.2.2 Metabolic Caging and Urine Protein Excretion

Rats were individually housed in metabolic cages for 24 hours, following a 1-2 hour habituation period. Standard rat chow and RO water were provided throughout the caging period. Urine volume was measured, and urine protein excretion was determined via absorbance using the benzethonium chloride method.
5.2.3 Glomerular Filtration Rate (GFR)

Assessment of GFR used an adaptation of the FITC-inulin protocol acquired from the Diabetic Complications Consortium (DiaComp): https://www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=28. An infusion of 3.74 µL/g of 5% FITC-inulin was infused into the tail vein with a 25G needle. A sample of 150 µL of blood was then collected in heparinized capillary tubes at 3, 7, 10, 15, 35, 55 and 75 minutes through tail vein collection with a fresh 25G needle. Samples were titrated by mixing 10 µL of plasma with 400 µL of 500 mM HEPES to maintain pH. Then, 50 µL of the titrated samples were pipetted into a 96 well plates. Fluorescence was assessed using a spectrophotometer set at 485 nm excitation and read at 538 nm emission.

5.2.4 Systolic Blood Pressure

Systolic blood pressure (SBP) was assessed by tail cuff plethysmography. Rats were warmed and monitored under a heat lamp for 10 minutes to promote vasodilation. A tail cuff and transducer were wrapped around the rat’s tail and inflated to a pressure of 200mmHg followed by slow deflation (Powerlab, ADInstruments, Colorado Springs, CO).

At sacrifice, kidney and body weights were collected and tissues were fixed in 10% NBF for paraffin embedding, flash frozen in liquid nitrogen or cryoembedded in OCT for biochemical assessment as described.
6 Histology

6.1 Tissue Sectioning

Tissues were formalin fixed at collection, processed and embedded in paraffin wax using a Leica TP1020 Tissue Processor and Leica EG1160 Paraffin Embedder respectively (Leica, Wetzlar, Germany). Blocks were chilled on wet ice for 30 minutes prior to sectioning on a Leica RM2145 Rotary Microtome. Blades were set at an angle of 3° and sections were cut into ribbons at a thickness of 3 µm. Ribbons were placed in a heated water bath to prevent wrinkling. Sections were separated and transferred onto charged microscope slides. Slides were dried at 37°C for 48 hours before use. Alternatively, tissues were cryoembedded in Tissue-Tek OCT compound (VWR, Radnor, PA), flash frozen in liquid nitrogen and stored at -80°C. OCT blocks were sectioned on a Leica cryostat CM 1900 at a thickness of 4 µM. Sections were transferred onto charged microscope slides and stored at -80°C until use.

6.2 Immunohistochemistry

Formalin fixed paraffin embedded rodent and human kidney sections were immunohistologically stained over a period of two days, as previously described (Advani et al., 2007). Slides were dewaxed by three consecutive incubations in xylene, three minutes each. Slides were then progressively hydrated in two washes of 100% ethanol, a single wash of 70% ethanol and three washes of distilled water for 3 minutes each. Antigen retrieval was achieved by submerging sections in citric acid buffer (Appendix), under boiling conditions for 10 minutes. Sections were cooled for 30 minutes before a single wash in PBS for five minutes. The sections were then
incubated in a solution of 3% H$_2$O$_2$ (Bio-Basics, Markham, ON, Canada) for 10 minutes, followed by two, five minute washes in PBS.

Next, a wax pen was used to draw a boundary around the kidney section, preventing spillage of the small volume of solution and ensuring maximum tissue coverage. Sections were incubated in two drops of serum-free protein blocking solution (Agilent Technologies) for 1 hour. Following incubation, sections were washed in three, 5 minute washes of PBS prior to antibody incubation. Sections were incubated in a 100 µL of primary antibody diluted in PBS at the desired concentration. Antibodies were prepared in the following concentrations: collagen IV 1:100 (Southern Biotech, Birmingham, AL) and p62 1:100 (Cell Signaling Technology). Incubation with an equivalent volume of PBS was used as the negative control. Sections were incubated at 4°C, overnight.

The following day, the primary antibody solution was washed off each section by three washes in PBS, 5 minutes each. The corresponding HRP-labelled polymer secondary antibody was added to each section and incubated at room temperature for 1 hour: anti-mouse (Agilent Technologies) and anti-rabbit (Agilent Technologies). After incubation, secondary antibodies were washed off as previously described and stained with liquid Diaminobenzidine and substrate chromogen system (DAB) (Agilent Technologies) for 10 minutes, and subsequently submerged in distilled water for 5 minutes. Sections were then counterstained in Mayer’s haematoxylin (Electron Microscopy Sciences, Hatfield, PA) for 1 minutes and flushed with running tap water to clear off excess stain. Then, sections were submerged in Scott’s tap water for five seconds, followed by distilled H$_2$O for 5 minutes. Finally, sections were dehydrated through successive incubations in
70% ethanol, three incubations in 100% ethanol and three incubations in xylene, for 3 minutes each. Sections were sealed by application of coverslips following the addition of a drop of dibutylphthalate polystyrene xylene (DPX) and air dried for 24 hours prior to analysis.

6.3 Histological Analysis

Immunohistologically stained kidney sections were scanned (Leica Microsystems Inc., Concord, ON, Canada) and analysed using Aperio’s ImageScope (Leica Microsystems Inc.) in a blinded manner. Estimation of glomerular collagen IV was measured as the proportion of positive immunostaining in 30 randomly selected glomeruli per section. Similarly, tubulointerstitial collagen IV was quantified as the proportional area of positive immunostaining in 10 randomly selected cortical fields at 100X. Positively stained tubules were defined as tubule cross-sections containing a minimum of one epithelial cell with cytoplasmic immunostaining for p62. The number of tubules positively staining for p62 was manually counted in 6-10 randomly selected 100X fields per section, and is represented as fold change relative to control. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) stain was conducted by the Pathology Research Program at Toronto General Hospital (Toronto, Ontario, Canada) and quantified as the number of TUNEL positive nuclei in 10 randomly selected cortical fields at a magnification of 100X.
7 Immunofluorescence

7.1 Fixation

NRK-52E cells were cultured on coverslips at a confluency of 50%. After treatment, cells were fixed in freshly prepared 2% paraformaldehyde (Electron Microscopy Sciences) in PBS (pH 7.2-7.4) for 12 minutes at room temperature. After fixation, cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) prepared in PBS for 3 minutes. Cryoembedded tissue sections were fixed and permeabilized after submersion in chilled 100% methanol for 10 minutes, followed by 3 washes of PBS, at 5 minutes each. Tissue sections were circled in wax pen as previously mentioned. Following fixation, immunofluorescent stain of cells and tissues was achieved through the same series of steps detailed below.

7.2 Immunofluorescent stain

Samples were incubated in 2% blocking buffer (Appendix) for 1 hour (1mL for cells, 100µL for tissue). Following incubation, blocking buffer was replaced with primary antibody, diluted in blocking buffer at the following concentrations: TFEB 1:100 (Abcam), p62 1:100 (Cell Signaling Technology), LAMP-1 1:100 (Cell Signaling Technology). For dual stains, both primary antibodies were added concurrently. The equivalent volume of blocking buffer was used for negative controls. Samples were incubated in primary antibody overnight, at 4°C.

The next day, primary antibodies were removed and samples were washed in PBS three times, 5 minutes per wash. The corresponding Alexa Fluor tagged secondary antibodies was prepared in
PBS and added to the samples in the following concentrations: Alexa Fluor 555 donkey anti-goat IgG 1:100 (Abcam) and Alexa Fluor 488 donkey anti-mouse IgG 1:100 (Abcam) and Alexa Fluor 555 donkey anti-rabbit IgG 1:100 (Abcam). Following a 1 hour incubation period, samples received 3 washes of PBS, at 5 minutes each. To visualize nuclei, DAPI (Cell Signaling Technologies) was applied at a concentration of 1:12,000 for two minutes, followed by two quick washes in PBS. Coverslips were sealed to charged glass slides by the addition of a single drop of fluoromount-aqueous mounting media (Sigma-Aldrich) and air dried in the dark for 24 hours prior to use.

7.3 Analysis of Immunofluorescent Staining

Images were collected on a Zeiss LSM700 Confocal microscope (Zeiss, Oberkochen, Germany) at a magnification of 630X, and fluorescently labelled antibodies were visualized at excitation lines 358 nm, 488 nm and 555 nm using Zen 2011 imaging software (Zeiss). Subsequently, channels were merged into a single image using Fiji ImageJ software (Schindelin et al., 2012) for analysis. Nuclear TFEB levels were quantified using Adobe Photoshop (CS4) (San Jose, CA). Nuclei were outlined using the lasso tool and histogram tool, and the ratio of red pixels to total pixels was used to indicate the relative amount of nuclear TFEB. In NRK-52E cells, TFEB nuclear translocation was assessed as the amount of red pixels in 6 randomly selected nuclei per 630X field.
8 Statistics

Statistical significance was determined by one-way ANOVA with a Fisher’s least significant difference test for comparison of multiple groups and paired or unpaired Student t test for comparison between two groups (or Mann-Whitney test for non-parametric data). Data were normalized to the average of the experimental controls and are expressed as mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA).
Chapter 4: Results

1 TFEB mRNA levels are diminished and p62-protein levels are increased in kidneys of patients with diabetic kidney disease

1.1 Clinical Characteristics of patients with diabetic kidney disease

Given the growing appreciation for the role of TFEB in upregulation of the autophagy-lysosomal pathway under conditions of cellular stress, and the paucity of information about TFEB expression levels in CKD, we set out to determine expression levels of TFEB in human diabetic kidney disease, the leading cause of CKD globally. For this experiment, we assessed archival biopsy tissue derived from 12 patients with histopathologically confirmed diabetic glomerulosclerosis and 12 individuals without diabetes. Tissue was obtained at the time of tumour nephrectomy along with tissue acquired from the unaffected region of the kidney. The mean age of the twelve patients without diabetes (controls) was 65±3 years, with eight out of twelve patients being male. All patients had an eGFR greater than 60 mL/min/1.73m². Six out of the twelve patients were hypertensive, with four out of the six individuals receiving treatment with an angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB). The mean age of patients with diabetic glomerulosclerosis was 67±2 years, with eight out of twelve patients being male. Eleven of the twelve patients had Type 2 diabetes and five patients experienced stage 3 CKD or worse as evidenced by an estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73m². Ten out of twelve patients were hypertensive, with seven out of those ten receiving treatment with either an ACEi or an ARB. RTqPCR assessment of TFEB mRNA levels in archival tissue from controls and people with diabetic kidney disease revealed an approximate
50% reduction in TFEB expression in kidney samples from people with diabetic kidney disease (Figure 1).

**Figure 1.** TFEB mRNA levels are diminished in kidney tissue from people with diabetic kidney disease. Real-time PCR quantitative assessment of fold change in renal TFEB mRNA expression in human kidney tissue from controls (h_Control, n=12) and people with diabetic kidney disease (h_Diabetes, n=12). AU=arbitrary units. *p<0.05.

1.2 p62 protein levels are increased in renal tubules from patients with diabetic kidney disease

To determine whether the downregulation in TFEB mRNA levels was associated with misfolded protein levels in renal cells, kidney sections were immunostained for the protein aggregate marker p62. There was an approximate 3 fold increase in the number of renal tubules immunopositive for p62 from patients with diabetic kidney disease relative to controls (Figure 2).
Figure 2. p62 levels increase in renal tubules of patients with diabetic kidney disease relative to control. (a) Representative photomicrographs of immunohistological stain for p62 and (b) quantitative assessment of fold change in the number of p62 positive tubules in kidney sections from patients without diabetic kidney disease (h_Control, n=10) and patients with diabetic kidney disease (h_Diabetes, n=10). Arrowheads identify p62 positive tubules. Scale bar=50 µm. *p<0.01.
2 Diminished TFEB expression and increased p62-protein aggregates are also a feature of chronic kidney disease in subtotally nephrectomized rats

2.1 Downregulation of TFEB mRNA is accompanied by increased p62 in kidneys from subtotally nephrectomized rats

Next, to gain mechanistic insight into the relationship between the downregulation of TFEB, p62 accumulation and kidney disease, we moved to an experimental model of chronic kidney disease reminiscent of the human condition, namely, the subtotally nephrectomized rat (SNx). Assessment of TFEB mRNA levels by RTqPCR revealed a reduction in TFEB mRNA levels in SNx rat kidneys comparable to the decline in TFEB expression observed in kidneys from patients with diabetic kidney disease (Figure 3). Likewise, immunostaining for p62 revealed an approximate two-fold increase in immunopositive p62-aggregates in tubule epithelial cells of SNx rats relative to sham-operated controls (Figure 4).
Figure 3. TFEB mRNA levels are diminished in the kidneys of subtotally nephrectomized rats relative to sham-operated controls. Real-time PCR quantitative assessment of fold change in renal TFEB mRNA expression in kidney homogenates from sham-operated (n=11) and subtotally nephrectomized (SNx) rats (n=12). AU=arbitrary units. *p<0.01.
Figure 4. p62 immunostaining is increased in subtotally nephrectomized rat kidneys relative to sham-operated controls as determined by immunohistological stain. (a) Representative photomicrographs of immunohistological stain and (b) quantitative assessment of fold change in the number of p62 positive tubules in kidney sections from sham-operated (Sham, n=10) and subtotally nephrectomized rats (SNx, n=8). Arrowheads identify p62 positive tubules. Scale bar=50 µm. *p<0.001.
Immunohistological stain for p62 uncovered an increase in p62 levels in renal tubule epithelial cells in both humans and rats with chronic kidney disease. To determine whether this observed increase in p62 was due to increased p62 expression, or an increase in p62-tagged protein aggregates, I set out on a series of experiments. In the first experiment, immunoblotting for p62 in kidney homogenates from sham and SNx rats revealed an increase in p62 levels in SNx rats (Figure 5).

![Figure 5](image)

**Figure 5.** p62 protein levels are increased in kidneys from subtotally nephrectomized rats compared to sham-operated controls, as determined by immunoblot. Representative immunoblot assessing p62 levels from sham (n=3) and subtotally nephrectomized (SNx, n=3) rat kidney homogenates. AU=arbitrary units. *p<0.05.

To determine whether the increase in p62 immunostaining was specific to p62 or a consequence of an increase in generalized protein misfolding, I probed for a secondary marker of misfolded proteins, namely, ubiquitin. Prior to p62 mediated aggregate formation, misfolded proteins that cannot be salvaged through chaperone mediated refolding are chemically modified with ubiquitin, targeting it for aggresome formation and bulk degradation through the UPS or autophagy-lysosomal pathway (Olzmann et al., 2008). Given the increase in p62 in SNx kidneys, I
hypothesized that I would also observe an increase in total ubiquitin levels, indicative of irreparable misfolded protein and a greater dependence on bulk degradative processes. To assess this, kidney homogenates from sham and SNx rats were immunoblotted for total ubiquitin levels. This assessment revealed an increase in total ubiquitin levels in SNx rat kidneys relative to sham-operated controls (Figure 6).

![Image](image-url)

**Figure 6.** Total ubiquitin levels are increased in subtotally nephrectomized rat kidneys relative to sham-operated rats. Representative immunoblot assessing total ubiquitin levels from sham (n=4) and subtotally nephrectomized (SNx, n=4) rat kidney homogenates. AU=arbitrary units. *p<0.05.

Next, reasoning that increased rates of protein misfolding often occur as a consequence of increased ER stress, I compared ER stress in kidneys from sham and SNx rats. For this experiment, I immunoblotted for the ER stress marker, phosphorylated eIF2α, in kidney homogenates from sham-operated and SNx rats. Upon sensing ER stress, membrane ER proteins initiate a series of pathways, one of which involves phosphorylation of the translation initiation factor eIF2α. In its phosphorylated form, eIF2α prevents translation, effectively halting new protein synthesis under conditions of ER stress (de Haro et al., 1996). Protein levels of
phosphorylated and total eIF2α were quantified by immunoblot and revealed an increase in phosphorylated eIF2α levels in SNx kidneys relative to control (Figure 7).

**Figure 7.** Protein levels of the endoplasmic reticulum stress marker phospho-eIF2α are increased in subtotally nephrectomized rat kidneys relative to sham-operated rats. Representative immunoblot and quantitative assessment of fold change in phosphorylated and total eIF2α in kidney homogenates from sham (n=3) and subtotally nephrectomized (SNx, n=3) rats. AU=arbitrary units. *p<0.001.

Lastly, it is appreciated that proximal tubules play a role in protein reabsorption from the urinary filtrate, introducing the possibility that the observed increase in protein aggregates in renal tubule epithelial cells could be due to increased reabsorption of urinary proteins under disease conditions. Reabsorbed proteins from the urinary filtrate are endocytosed into vesicles that then fuse with lysosomes where they are hydrolyzed to their constituent amino acids (Nielsen, 1994). Therefore, to exclude this possibility, I conducted a dual immunofluorescence stain for p62 and the lysosomal membrane protein LAMP-1 in sham and SNx rat kidneys. Whereas we observed a marked increase in p62 labelled aggregates in the tubules of SNx kidneys relative to control, these aggregates did not co-localize with LAMP-1, indicating that the observed protein aggregates were not concentrated in lysosomes, and therefore, were likely endogenous in origin (Figure 8).
Figure 8. p62 does not co-localize with the lysosomal membrane protein LAMP-1 in rat kidney tubule epithelial cells. Immunofluorescence microscopy for p62 and LAMP-1 in cryoembedded kidney sections from sham-operated (Sham) and subtotally nephrectomized (SNx) rats depicting little co-localization between p62 tagged protein aggregates (arrowheads) and LAMP-1 labelled lysosomes (arrows). Scale bar=15 µm.

In summary, our study of kidney disease from humans with CKD caused by diabetes and in rats with CKD caused by renal ablation, revealed a consistent downregulation of TFEB and an associated increase in p62-tagged misfolded protein aggregates. In considering mechanisms to remedy these features, we wondered whether HDAC6 inhibition could alter TFEB activity.
3 HDAC6 inhibition increases TFEB acetylation and nuclear localization in NRK-52E cells

3.1 Tubastatin A administration increases acetylation of the HDAC6 substrate α-tubulin in NRK-52E cells

To determine whether HDAC6 inhibition alters TFEB activity, I treated proximal tubule lineage NRK-52E cells with the small molecule HDAC6 inhibitor Tubastatin A. In initial experiments to determine the efficacy of Tubastatin A in NRK-52E cells, a dose response experiment was performed by exposing cells to increasing concentrations of Tubastatin A before immunoblotting for acetylated α-tubulin, a known substrate of HDAC6. Taking this approach, I observed a dose-dependent increase in acetylated α-tubulin following treatment with Tubastatin A (Figure 9). Although specific for HDAC6 (IC$_{50}$=15 nM), it has been suggested that higher doses of Tubastatin A (10 µM) may also inhibit other HDAC isoforms (Butler et al., 2010a; Oehme et al., 2013). Therefore, I opted to use a concentration of 2.5 µM. This selection is consistent with previous literature that has demonstrated effective HDAC6 inhibition at this dose (Butler et al., 2010a).
Figure 9. Tubastatin A induces a dose-dependent increase in acetylated α-tubulin levels in NRK-52E cells. Immunoblot and quantitative assessment for acetylated and total α-tubulin in NRK-52E cells (n=3/group) treated with increasing doses of Tubastatin A at the following concentrations: 0µM, 0.6µM, 1.2µM, 2.5µM, 5µM and 10µM over a period of 24 hours. AU=arbitrary units. *p<0.01 vs. control, †p<0.001 vs. control, ‡p<0.0001 vs. control, §p<0.05 vs. 0.6µM, ¶p<0.05 vs. 1.2µM, ||p<0.0001 vs. 0.6µM, **p<0.01 vs. 1.2µM, ††p<0.01 vs. 2.5µM.

3.2 HDAC6 inhibition increases TFEB acetylation

Next, having observed that Tubastatin A increased the acetylation status of the known HDAC6 substrate, α-tubulin, I wondered if Tubastatin A could alter the acetylation status of TFEB. For this experiment, NRK-52E cells were incubated with 2.5 µM of Tubastatin A for 24 hours. Then, immunoprecipitation for TFEB was conducted. By immunoblot, I assessed levels of acetylated
lysine, the amino acid known to be deacetylated by HDAC6. It was found that under basal conditions, TFEB is acetylated, and that levels of TFEB lysine acetylation increased following treatment with Tubastatin A (Figure 10).

**Figure 10.** Tubastatin A increases TFEB acetylation in NRK-52E cells. Immunoblot and quantitative assessment for acetylated lysine in NRK-52E cells (n=3/group) subjected to immunoprecipitation for TFEB following a 24 hour incubation period with vehicle or 2.5 µM Tubastatin A. AU=arbitrary units. *p<0.05.
3.3 HDAC6 inhibition increases TFEB nuclear translocation and transcriptional activity in NRK-52E cells

We next set out to determine whether increased TFEB acetylation following HDAC6 inhibition with Tubastatin A could alter TFEB activity. TFEB activity is regulated by its cellular localization. Whereas TFEB resides in the cytosol under basal conditions, the induction of cellular stress causes an increase in the nuclear translocation of TFEB. Therefore, to assess the functional consequence of HDAC6 inhibition on TFEB activity, I treated NRK-52E cells with 2.5 µM of Tubastatin for 24 hours. By immunoblot of the cytosolic and nuclear fraction, and immunofluorescent stain for co-localization of TFEB with the nuclear marker 4’6-diamino-2-phenylindole (DAPI), we observed an increase in the proportion of nuclear TFEB in NRK-52E cells treated with Tubastatin A (Figure 1). This increase in nuclear translocation of TFEB was accompanied by an approximate 30% increase in mRNA levels of the TFEB target LAMP-1 (LAMP-1 mRNA:RPL13a mRNA [arbitrary units]: Control, 1.0±0.0; Tubastatin A 1.3± 0.1. p<0.05).
Figure 11. Tubastatin A increases TFEB nuclear localization in NRK-52E cells. (a) Immunoblotting for nuclear TFEB in NRK-52E cells treated with vehicle or 2.5 µM Tubastatin A for 24 hours (n=3/group). (b) Immunofluorescence staining for nuclear TFEB in NRK-52E cells treated with vehicle (n=6) or 2.5 µM Tubastatin (n=9) for 24 hours. Scale bar= 15 µm. *p<0.05, †p<0.001.

3.4 Tubastatin A prevents programmed cell death in NRK-52E cells

Next, to determine whether the increase in TFEB nuclear translocation following HDAC6 inhibition affects cell survival, assays for programmed cell death were performed following exposure of cells to conditions of increased endoplasmic reticulum stress. In cultured NRK-52E cells, 500 nM of the ER stress inducer thapsigargin was administered following pre-treatment with 2.5 µM Tubastatin A. Then, samples were immunoblotted for cleaved caspase-3 or subjected to flow cytometric assessment of annexin V labelling. Cleavage of caspase-3 leads to its
activation of downstream signalling that culminates in apoptosis. Because of its essential role, it is an established marker of programmed cell death. Similarly, annexin V is a calcium-dependent phospholipid that binds to the exposed phospholipid phosphotidylserine (PS) on the surface of cells undergoing programmed cell death. As such, an increase in annexin V positive cells is indicative of a greater proportion of cells undergoing programmed cell death. Whereas cells incubated with the ER stress inducer thapsigargin had a greater proportion of cells undergoing programmed cell death, pre-treatment of NRK-52E cells with Tubastatin A prior to incubation with thapsigargin significantly reduced levels of cleaved caspase 3 (Figure 12). Similarly, Tubastatin A significantly reduced the amount of early-apoptotic cells (annexin V+/ 7AAD-) (Figure 13) and necrotic cells (annexin V+/7-AAD+ cells [%]: Control, 2.3±0.4; Tubastatin A 2.5±0.1; thapsigargin, 2.8±0.4; *thapsigargin + Tubastatin A, 1.6±0.1. *p<0.05 vs. thapsigargin) following exposure to thapsigargin.
Figure 12. Tubastatin A attenuates programmed cell death in NRK-52E cells as assessed by cleaved caspase-3. Immunoblot and quantitative assessment of cleaved caspase 3 protein levels in NRK-52E cells treated with 500 nM of the ER stress inducer thapsigargin for 24 hours following preincubation with 2.5 μM Tubastatin A or respective controls. *p<0.05 vs. thapsigargin + vehicle.
Figure 13. Tubastatin A attenuates programmed cell death in NRK-52E cells as assessed by annexin V positive staining. Flow cytometric analysis of annexin V staining in NRK-52E cells treated with 500 nM of the ER stress inducer thapsigargin for 24 hours following preincubation with 2.5 µM Tubastatin A or respective controls. *p<0.01 vs. control, ‡‡p<0.05 vs. Tubastatin A, §§p<0.05 vs. thapsigargin.
4 Tubastatin A administration attenuates progressive proteinuria and structural remodelling in experimental CKD

4.1 Tubastatin A inhibits HDAC6 in rat kidneys

Having identified that HDAC6 inhibition increases nuclear translocation of TFEB and improves tubule epithelial cell viability under conditions of ER stress in-vitro, we next sought to determine the effect of Tubastatin A on renal function in a rodent model of advanced chronic kidney disease, namely, the SNx rat model. Given the downregulation of TFEB in kidneys from SNx rats, we queried whether adapting a method of increasing TFEB nuclear translocation and activity through HDAC6 inhibition would serve a renoprotective role. In initial experiments, sham rats were randomized to receive a subcutaneous (s.c) injection of either vehicle (5% dextrose) or Tubastatin A (30mg/kg) administered thrice weekly for a period of three weeks. By immunoblot, we found that this dose induced an increase in acetylated α-tubulin levels in rat kidney homogenates, thus confirming the efficacy of the small molecule inhibitor Tubastatin A in inhibiting renal HDAC6 at the selected dosing regimen (Figure 14).
Figure 14. Tubastatin A increases acetylated α-tubulin levels in rat kidney homogenates. Immunoblot and quantitative assessment for acetylated and total α-tubulin in kidney homogenates from rats treated with vehicle (n=5) or 30 mg/kg s.c injection of Tubastatin A (n=6), administered thrice weekly over three weeks. AU=arbitrary units. *p<0.05.
4.2 Tubastatin A attenuates progressive proteinuria in subtotally nephrectomized rats

Next, renal ablation or sham surgery was conducted on male Sprague Dawley rats to generate sham-operated or SNx rats. Four weeks after surgery, assessment of urine protein levels was conducted to confirm the onset of kidney dysfunction. Then, rats were randomized to receive vehicle or Tubastatin A (30 mg/kg s.c) thrice weekly for the remaining three weeks of the study. A detailed outline of the study design is summarized in the Figure 15.

Figure 15. Flow diagram of in-vivo pharmacological study of HDAC6 inhibition in subtotally nephrectomized rats (SNx). Male Sprague Dawley (age 8 weeks) underwent sham or subtotal (5/6) nephrectomy. Rats were studied for 4 weeks before the assessment of urine protein, and then randomized to receive Tubastatin A (30 mg/kg in 5% dextrose by thrice weekly subcutaneous injection) or vehicle for three weeks. Then, urine protein levels and functional parameters (glomerular filtration rate, systolic blood pressure, body weight) were assessed (7 weeks post-surgery) prior to collection of kidneys for biochemical assessment.

After surgery, proteinuria was measured as an indicator of declining renal function. Four weeks following surgery, SNx rats displayed a four fold increase in their urine protein relative to sham-operated rats (Figure 16a). After establishing comparable renal damage between those rats that
had received renal ablation surgery, rats were randomized to receive either vehicle or 30 mg/kg (s.c) of Tubastatin A for the remaining three weeks of the study. At the study’s conclusion, proteinuria was once again measured. Whereas SNx rats receiving vehicle showed an approximate doubling of their urine protein by week seven, those rats that received Tubastatin A show a stabilization of their urine protein, comparable to their four week levels (Figure 16b).
Figure 16. Tubastatin A attenuates progressive proteinuria in subtotally nephrectomized rats. (a) Urinary protein excretion in sham and subtotally nephrectomized (SNx) rats four weeks after surgery and before the initiation of treatment. (b) Urinary protein excretion in sham and SNx rats treated with vehicle or 30mg/kg s.c. Tubastatin A administered thrice weekly for the remaining three weeks of the seven week study. *p<0.05 vs. sham + vehicle, †p<0.05 vs. sham + Tubastatin A, ‡p<0.01 vs. sham + Tubastatin A, §p<0.05 vs. SNx + vehicle.
4.3 Physiological parameters of sham and subtotally nephrectomized rats treated with vehicle or Tubastatin A

At the end of the study period, measures of kidney function (Table 1) were assessed, and renal tissue was harvested from all rats for structural analysis. SNx surgery resulted in a significant decrease in body weight relative to sham-operated rats, with Tubastatin A causing mild decreases in body weight in both sham and SNx rats. In addition, SNx surgery led to a significant increase in remnant kidney weight, whereas Tubastatin A administration prevented renal enlargement in SNx rats, with final kidney weights being comparable to sham-operated rats receiving vehicle. By the end of the study, SNx rats displayed pathophysiological changes resulting from progressive renal decline as evidenced by a significant increase in systolic blood pressure (SBP) and a significant decrease in glomerular filtration rate (GFR) (Table 1). Whereas Tubastatin A led to very mild improvements in SBP and GFR in SNx rats, these changes were not statistically significant.
Table 1. Functional characteristics of sham-operated and subtotally nephrectomized (SNx) rats treated with vehicle or Tubastatin A.

<table>
<thead>
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<th>Body weight (g)</th>
<th>Left kidney weight (g)</th>
<th>Left kidney:body weight (%)</th>
<th>SBP (mmHg)</th>
<th>GFR (ml/min/kg)</th>
</tr>
</thead>
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<tr>
<td>Sham + vehicle</td>
<td>610±20</td>
<td>1.69±0.06</td>
<td>0.28±0.01</td>
<td>126±2</td>
<td>8.5±0.5</td>
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<tr>
<td>Sham + Tubastatin A</td>
<td>562±16*</td>
<td>1.56±0.04</td>
<td>0.28±0.01</td>
<td>123±2</td>
<td>8.8±1.0</td>
</tr>
<tr>
<td>SNx + vehicle</td>
<td>516±11*</td>
<td>2.42±0.07‡§</td>
<td>0.47±0.01‡§</td>
<td>204±12‡§</td>
<td>2.8±0.7‡§</td>
</tr>
<tr>
<td>SNx + Tubastatin A</td>
<td>513±15†</td>
<td>1.86±0.10§‖</td>
<td>0.36±0.02§‖</td>
<td>189±12§</td>
<td>3.3±0.3§</td>
</tr>
</tbody>
</table>

SBP = systolic blood pressure, GFR = glomerular filtration rate

*p<0.05 vs. sham + vehicle, †p<0.001 vs. sham + vehicle, ‡p<0.0001 vs. sham + vehicle, §p<0.0001 vs. sham + Tubastatin A, ¶p<0.01 vs. sham + Tubastatin A, ||p<0.0001 vs. SNx + vehicle.
4.4 Tubastatin A attenuates tubulointerstitial, but not glomerular, collagen IV deposition

Next, to assess the effect of Tubastatin A on structural remodelling, we immunohistologically stained for collagen IV in formalin fixed paraffin embedded kidney sections from sham-operated and SNx rats that received treatment with either vehicle or Tubastatin A (Figure 17). Tubulointerstitial fibrosis was quantified as the proportional area of cortical tubulointerstitial collagen IV immunostaining. No observable histological changes in collagen IV deposition were observed in sham-operated animals treated with Tubastatin A relative to vehicle. Renal ablation significantly increased the degree of cortical tubulointerstitial fibrosis relative to sham-operated rats. However, whereas SNx rats treated with vehicle had a four-fold increase in the proportional area of collagen IV immunostaining, SNx rats treated with Tubastatin A only showed a two-fold increase in collagen IV immunostaining relative to sham-operated animals. Similarly, renal ablation also resulted in significant glomerular fibrosis as evidenced by a three-fold increase in glomerular collagen IV deposition in SNx rats relative to sham-operated rats. However, whereas Tubastatin A was associated with a very mild decrease in glomerular collagen IV in SNx rats, this reduction was not statistically significant.
Figure 17. Immunohistological stain for collagen IV in sham and subtotally nephrectomized rats treated with vehicle or Tubastatin A. Representative photomicrographs and quantification of immunohistological stain for (a) tubulointerstitial collagen IV and (b) glomerular collagen IV in sham or subtotally nephrectomized (SNx) rats treated with vehicle or Tubastatin A. (Sham: vehicle, n=10; Tubastatin A, n=10. SNx: vehicle, n=6; Tubastatin A, n=10). Scale bar= 50 μm. *p<0.05 vs. sham + vehicle, †p<0.05 vs. sham + Tubastatin A, ‡ p<0.05 vs. SNx + vehicle.
4.5 Tubastatin A increases nuclear translocation of TFEB and reduces p62 accumulation in-vivo

Having discovered that HDAC6 inhibition by Tubastatin A attenuates functional and structural decline in SNx rats, I set out to determine if this was associated with an increase in TFEB activity as seen in our in-vitro experiments. To assess this, the proportion of nuclear TFEB was measured by immunoblot on nuclear fractions isolated from kidney homogenates from sham and SNx rats treated with vehicle or Tubastatin A. Consistent with our in-vitro findings, HDAC6 inhibition by Tubastatin A increased the proportion of nuclear TFEB in kidneys of sham and SNx rats relative to their vehicle treated counterparts (Figure 18a). Having demonstrated that HDAC6 inhibition increased TFEB translocation both in-vitro and in-vivo, we hypothesized that an increase in TFEB activity would be accompanied by a reduction in p62 accumulation in kidney tubules of SNx rats. To assess this, formalin-fixed paraffin embedded kidney sections from sham and SNx rats treated with vehicle or Tubastatin A were immunohistologically assessed for the protein aggregate marker p62. Similar to kidneys from patients with diabetic kidney disease, and our initial screen of SNx kidneys, there was a significant increase in p62-labelled aggregates in renal tubules of SNx rats. Interestingly, treatment with Tubastatin A was accompanied by a marked reduction in p62 immunostaining in renal tubules (Figure 18b and 18c).
Figure 18. Tubastatin A increases nuclear localization of TFEB which is accompanied by a reduction in p62-labelled protein aggregates in the kidneys of subtotally nephrectomized rats. (a) Immunoblot for TFEB on nuclear and cytosolic fractions of kidney homogenates from sham and subtotally nephrectomized (SNx) rats treated with vehicle or Tubastatin A. Immunoblot is representative of at least three samples per group. (b) Representative photomicrographs and quantification of immunohistological stain for p62 in sham or SNx rats treated with vehicle or Tubastatin A. Arrow heads point to p62 positive tubules. (Sham: vehicle, n=10; Tubastatin A, n=10. SNx: vehicle, n=8; Tubastatin A, n=10). Scale bar=50 µm. *p<0.001 vs. sham + vehicle. †p<0.001 vs. sham + Tubastatin A. ‡p<0.05 vs. sham + Tubastatin A. §p<0.001 vs. SNx + vehicle.
4.6 Tubastatin A attenuates tubule epithelial cell death in subtotally nephrectomized rats

In my final series of experiments, I set out to assess the renoprotective role of Tubastatin A on tubule epithelial cell viability. Formalin fixed, paraffin embedded kidney sections from sham-operated and SNx rats treated with vehicle or Tubastatin A were assessed for cell death using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). As expected, SNx rats displayed an increase in tubule epithelial cell death relative to their sham-operated counterparts. However, SNx rats receiving treatment with Tubastatin A had less TUNEL positive nuclei (Figure 19).
Figure 19. Tubastatin A reduces the number of TUNEL positive nuclei in subtotally nephrectomized rats. Representative photomicrographs and quantification of TUNEL stain in sham or subtotally nephrectomized (SNx) rats treated with vehicle or Tubastatin A. Arrow heads point to TUNEL positive nuclei. (Sham: vehicle, n=9; Tubastatin A, n=9. SNx: vehicle, n=8; Tubastatin A, n=9). *p<0.001 vs. sham + vehicle, †p<0.001 vs. sham + Tubastatin A, ‡ p<0.05 vs. SNx + vehicle.
Chapter 5
Discussion

1 Overview

In this study, I found that TFEB downregulation was associated with an increase in p62-labelled aggregates in human and rodents with diabetic and experimental CKD, highlighting misfolded protein accumulation as a feature of chronic kidney disease. Secondly, I found that HDAC6 inhibition by Tubastatin A increased TFEB activity through alteration of its acetylation status. Increased TFEB acetylation following Tubastatin A treatment was associated with increased TFEB nuclear localization and induction of TFEB transcriptional activity. Induction of TFEB activity correlated with a reduction in tubule epithelial cell death. In-vivo, HDAC6 inhibition by Tubastatin A attenuated progressive proteinuria and blunted structural remodelling. This was associated with increased nuclear translocation of TFEB in renal tissue from rats treated with Tubastatin A and was accompanied by both improved clearance of misfolded protein aggregates in renal tubules, and improved tubule cell viability. Collectively, this study highlights HDAC6 inhibition as one method of inducing TFEB activity which may provide a new treatment option for CKD.
2 TFEB downregulation is associated with increased p62-accumulation in human diabetic kidney disease

In the first series of experiments, samples of human cortical kidney tissue from patients with and without histopathologically confirmed diabetic glomerulosclerosis were assessed for changes in TFEB expression. While the use of archival human kidney tissue in this manner provides the strength of assessing pathological processes within an intrinsically variable patient population, we are cognizant of a number of issues that can arise from its use. For example, in this study, samples were acquired during tumour nephrectomy which is a process that requires surgically induced renal clamp ischemia prior to acquisition of the biopsy. In addition to in-vivo ischemic conditions, once acquired, the sample is subjected to ex-vivo ischemic conditions during collection, storage and transport before fixation. Studies have shown that this period is associated with degradation of RNA and a change in gene expression in the isolated tissue, namely, in the induction of hypoxia inducible factors, and upregulation of apoptotic pathways (Sun et al., 2016). Since we are evaluating changes in gene expression, ischemia induced perturbations in transcription could serve as a potential confounding variable. However, kidney tissue from patients without diabetes that served as controls were acquired under similar conditions and would be expected to have comparable alterations in gene expression, a factor that would be minimized upon normalization. In our isolation of RNA, we opted to acquire RNA from paraffin embedded renal tissue using practices that are already established in our lab (Advani et al., 2009). To account for the risk of RNA degradation, I assessed the integrity of an isolated RNA sample from paraffin embedded tissue and found that the isolated RNA had a RNA integrity number (RIN) of 7.20, indicating suitable RNA quality for our downstream assessments (Schroeder et al., 2006).
Accepting the limitations of experimentation in archival formalin-fixed paraffin embedded tissue, quantitative assessment of TFEB expression levels by RTqPCR revealed that TFEB was downregulated in kidney tissue from humans with diabetic kidney disease. Suggestive of a downregulation of autophagy, we postulated that this would manifest as an increase in misfolded protein accumulation. Therefore, an immunohistological stain was conducted for p62, a protein aggregate marker known for its role in sequestering misfolded proteins into aggregates for bulk degradation through the autophagy-lysosomal pathway. Immunohistological assessment revealed a marked and consistent increase in p62-labelled aggregates in renal tubules of patients with diabetic kidney disease relative to controls. This finding is consistent with other studies that have noted an increase in p62 aggregates in the tubules of obese mice with marked renal dysfunction (Yamahara et al., 2013), but the contribution of these protein aggregates to the progression of kidney disease remains unclear.

Clinically, dysregulation in TFEB and p62 accumulation seems to precede major reductions in GFR, with most of the patients with diabetic kidney disease in the present study having an eGFR of greater than 60 mL/min/1.73m². In its present form however, this study is mechanistically limited and unable to establish a temporal or causal relationship between TFEB decline and impaired renal function. Since proteinuria precedes GFR decline in earlier stages of disease (Regeniter et al., 2009), it would be interesting to assess the correlation between proteinuria and the onset of TFEB decline in the earlier stages of the disease (stage 1-2), as well as the endurance of TFEB decline and protein aggregates in more advanced stages of CKD associated with greater declines in GFR (stage 4-5). Unfortunately, historical urine protein data were not available for some of the patients from whom kidney tissue had been obtained. Secondly, most patients with diabetic kidney disease received blood pressure lowering therapy through an ACEi or ARB. It is
unknown whether the changes in TFEB gene expression and protein aggregation could be influenced by these medications and thus presents as a potential confounder. Given these limitations, and our interest in gaining mechanistic insight into the relationship between TFEB and CKD, we moved to assess TFEB levels and protein aggregation in an experimental model of CKD.

3 Diminished TFEB and increased misfolded protein accumulation occur in subtotally nephrectomized rats

In selecting a model of experimental CKD, we chose to assess TFEB levels and protein aggregation in SNx rats, a model of progressive proteinuric kidney disease generated through renal ablation. The reduction in renal mass in SNx rats is accompanied by hyperfiltration, proteinuria, compensatory growth, glomerular and tubulointerstitial fibrosis and GFR decline reminiscent of human CKD including diabetic kidney disease even though rats are normoglycemic (Kaufman et al., 1974). Correspondingly, most rodent models of diabetes do not develop GFR decline, and do not mimic the tubulointerstitial injury increasingly appreciated as a key contributor to human diabetic kidney disease (Gilbert, 2017). In the SNx model, we also observed downregulation of TFEB and an accumulation of p62 immunostaining in tubules of SNx rats, highlighting these features as common factors of CKD.

The downregulation of TFEB and the accumulation of p62 in renal tubules was suggestive of increased protein misfolding and impaired autophagic clearance of misfolded proteins through dampening of the autophagy-lysosomal pathways. However, unsure as to whether the increase in
p62 was reflective of increased p62 accumulation or p62 expression, I blotted kidney homogenates from sham and SNx rats for p62, which revealed a marked increase in p62 in diseased rat kidneys. Whereas this may be suggestive of impaired clearance of p62-tagged proteins, the use of p62 as a sole marker of misfolded protein aggregation is limited due to the fact that p62 is itself, a transcriptional target of TFEB, and is known to participate in other signalling cascades (Ivankovic et al., 2016). Therefore, to further assess the degree of misfolded protein accumulation, I moved upstream to assess the degree of endoplasmic reticulum stress (ER stress), a causal factor in increased rates of protein misfolding that often precedes the accumulation of misfolded protein aggregates. I blotted kidney homogenates from sham and SNx rats for the ER protein phospho-eIF2α and found a significant increase in this marker of ER stress in SNx kidneys. The finding of increased ER stress in the diseased kidney is consistent with a growing body of literature that points to ER stress as a key cellular perturbation in the pathogenesis of kidney disease, and has been observed in multiple cases of kidney injury, including proteinuria (Ohse et al., 2006), hyperglycemia and the development of AGEs in the urinary filtrate (Lindenmeyer et al., 2008), and uremic toxins (Kawakami et al., 2010).

Under conditions of ER stress, chaperones are employed as a first line measure to re-fold proteins into their proper configurations (Hetz, 2012). When the demands of re-folding surpass the ability of chaperones to salvage proteins, proteins are tagged with ubiquitin for bulk degradation (Olzmann et al., 2008). Therefore, to further validate whether the increase in p62 was reflective of increased misfolded protein accumulation, I immunoblotted kidney homogenates for ubiquitin and observed an increase in total ubiquitin levels in SNx rat kidneys relative to sham rats, supporting the presence of irreparable, misfolded proteins that have not been degraded by the proteasome or autophagy-lysosomal pathway.
Having confirmed the presence of misfolded protein aggregates in renal tubules of SNx rats, and cognizant of the vital role of proximal tubules in protein reabsorption, I queried whether the increase in protein aggregation was reflective of protein uptake from the urinary filtrate, for which, reabsorbed proteins fuse with lysosomes for degradation into their constituent amino acids (Nielsen, 1994). To assess this, I probed for the lysosomal marker LAMP-1 and p62 and found no colocalization, indicating that the aggregates of misfolded proteins originated from within the cell. Collectively, these findings indicate that TFEB expression is diminished in CKD and coincides with an increase in misfolded protein accumulation.

As a master regulator of autophagy, TFEB remains bound to activated mTORC1 and its binding partner 14-3-3, in the cytosol. Upon nutrient deprivation, mTORC1 is inactivated, allowing for TFEB de-phosphorylation, subsequent release from 14-3-3 and its translocation to the nucleus where it can induce 1) upregulation of the CLEAR network and 2) its own self-inducible transcription (Martini-Stoica et al., 2016). The apparent downregulation of TFEB in diabetic kidney disease is consistent with previous research noting hyperactive mTORC1 in CKD. Hypothetically, this would effectively sequester TFEB in the cytosol and prevent TFEB mediated autophagy-lysosomal pathway upregulation, thus dampening the autophagic response. Interestingly, the downregulation of TFEB, which may be a contributing factor in the downregulation of autophagy, may also contribute to the accumulation of misfolded protein by way of its regulation of the UPR. TFEB plays a role in the integrated stress response and upregulates the responsiveness of the UPR to misfolded proteins by increasing levels of ATF4, a transcription factor that increases expression of genes involved in the unfolded protein response.
(Martini-Stoica et al., 2016). It is plausible that the increase in protein aggregates could be due to a number of circumstances, starting with an increase in ER stress, and accompanied by downregulation of both the UPR and autophagy systems, resulting in the accumulation of misfolded proteins in the cytosol. Furthermore, ubiquitinated protein aggregates in the cytoplasm participate in a feedback loop to the UPS, reducing the efficiency of the proteasome, further precipitating an increase in accumulated misfolded protein (Kopito, 2000).

Given the finding that misfolded protein accumulation is a feature of human and experimental CKD, identifying methods to increase TFEB activity may hold therapeutic promise in increasing clearance of protein aggregates. While the most obvious method of achieving this goal is modulation of TFEB phosphorylation through inhibition of mTORC1, the use of mTORC1 inhibitors is itself associated with adverse renal outcomes (Diekmann et al., 2012). Recent work by Bao and colleagues has identified novel TFEB regulation through acetylation, suggesting mTORC1 independent mechanisms of regulation (Bao et al., 2016). Therefore, we set out to assess modulators of TFEB acetylation status as potential regulators of its activation.
4 HDAC6 inhibition induces TFEB activity in NRK-52E cells

We opted to study HDAC6 as a potential regulator of TFEB acetylation and activation. As a cytosolic deacetylase, HDAC6 imparts regulation of transcriptional activity through regulation of nuclear shuttling of a number of transcription factors. For example, under basal conditions HDAC6 forms a tri-complex with HSF1 and HSP90, effectively sequestering HSF1 in the cytosol (Boyault et al., 2006b). To assess the effect of HDAC6 inhibition on TFEB, I first tested the efficacy of a small molecule inhibitor of HDAC6, Tubastatin A in NRK-52E cells, an immortalized cell line of proximal tubule lineage. Tubastatin A is highly selective for the DD2 domain of HDAC6 as evidenced by an IC\textsubscript{50} of 0.015 µM±0.001 (Butler et al., 2010a). Incubation of NRK-52E cells with increasing concentrations of Tubastatin A led to a dose-dependent increase in hyperacetylation of the HDAC6 substrate α-tubulin. However, a key limitation of the use of small molecule inhibitors is the potential off-target inhibition of other isoforms. Indeed, while Tubastatin A has an IC\textsubscript{50} of greater than 30 for most HDAC isoforms, it has an IC\textsubscript{50} of 0.0854 µM±0.040 for HDAC8 (Butler et al., 2010a), suggesting its potential inhibition at higher doses. Furthermore, when administered at 10 µM in a separate study, Tubastatin A led to acetylation of histones, indicative of class I HDAC inhibition (Butler et al., 2010a). Administration of Tubastatin A at 2.5 µM has been found to induce α-tubulin without the acetylation of histone, indicative of HDAC6 specific inhibition at this dose (Butler et al., 2010a). Therefore, I opted to use a dose of 2.5 µM for all in-vitro experiments conducted in this study.

By way of immunoprecipitation in NRK-52E cells, I found that TFEB is a direct substrate for HDAC6 mediated deacetylation. HDAC6 inhibition led to hyperacetylation of TFEB at its lysine residues and greater nuclear localization following treatment with Tubastatin A. However, given
the potential for off-target effects of the small molecule inhibitor, in future experiments, I would seek to recapitulate these findings by administering a small interfering RNA duplex (siRNA) designed for degradation of HDAC6 mRNA. Following knockdown of HDAC6, I would assess acetylation and nuclear localization of TFEB to confirm that the changes we observed were governed by HDAC6. In spite of this limitation, increased acetylation and nuclear localization of TFEB following Tubastatin A treatment suggests that HDAC6 may participate in sequestering TFEB in the cytosol.

The mechanism by which increased TFEB acetylation contributes to its nuclear localization is unknown. Previous literature has shown that HDAC6 inhibition blocks the interaction between 14-3-3 proteins and its binding partners (Mortenson et al., 2015). It is possible that acetylation could change the electrostatic interaction between TFEB and its cytosolic binding partner 14-3-3, potentially weakening their interaction and promoting the nuclear translocation of TFEB. In future experiments, I could assess this by conducting mass spectrometry analysis of acetylated residues following both pharmacological inhibition and knockdown of HDAC6. Then, by way of site directed mutagenesis at the identified acetylation sites, I could assess 14-3-3’s binding affinity to acetylation resistant TFEB mutants and hyperacetylated TFEB mutants to gain insight into the mechanism behind the association between TFEB acetylation and its increased nuclear translocation.

Next, to assess the functional consequence of TFEB hyperacetylation and nuclear translocation, I quantified gene expression changes in a TFEB transcriptional target known to change during induction of the autophagy-lysosomal pathway. Tubastatin A led to upregulation of the lysosomal
protein LAMP-1 mRNA levels, suggestive of CLEAR network activation. To further validate this finding, in future experiments, it would be prudent to assess transcriptional changes in multiple CLEAR network genes. Secondly, to determine a causal relationship between HDAC6 inhibition and subsequent TFEB activation of transcriptional networks, one would knockdown TFEB using siRNA and assess changes in LAMP-1 expression following incubation with Tubastatin A. Nonetheless, the observation that Tubastatin A induced upregulation of LAMP-1 suggests that HDAC6 inhibition may upregulate the autophagy-lysosomal pathway, plausibly through an acetylated TFEB dependent mechanism.

Whereas the acetylation of TFEB has been reported by Bao and colleagues, they found that TFEB deacetylation by the Class III HDAC SIRT1 increased nuclear translocation, and subsequent activation of CLEAR network genes (Bao et al., 2016). Our finding that hyperacetylation leads to a comparable result indicates that HDAC6 may act at a different residue. Future work using mass spectrometry assessment of acetylated residues following HDAC6 inhibition may provide insight as to its site of action. Interestingly, Bao and colleagues only reported partial changes in CLEAR network activation, but not upregulation of the full spectrum of CLEAR network genes. It would be interesting to determine whether hyperacetylation of TFEB imparts preferential binding to different promoter regions, which could lead to partial upregulation of portions of the autophagy lysosomal pathway, such as late stage lysosomal biogenesis as we observed, and not all TFEB mediated genes. Indeed, other transcription factors, e.g. Kruppel-like factor 4 (Klf-4), demonstrates preferential binding based on post-translational modifications in which methylation sites increase the binding affinity of Klf-4 to CpG sites in promoter regions (Hashimoto et al., 2016). Whether acetylation imparts preferential binding to promoter regions in a similar fashion may be a topic for future study. One means to assess this would be to conduct a chromatin
immunoprecipitation (ChIP) experiment following Tubastatin A treatment in NRK-52E cells and assess for enrichment of acetylated TFEB at multiple promoters to assess for preferential enrichment for certain genes relative to the entire CLEAR network.

5 Tubastatin A prevents programmed cell death in NRK-52E cells

To assess downstream effects of increased TFEB activity, we opted to study changes in programmed cell death in proximal tubule cells. This is because proximal tubule cell programmed cell death has been widely associated with CKD development in the literature (reviewed in (Schelling, 2016). Proximal tubule cells were subjected to ER stress based on the finding that ER stress was increased in diseased SNx rat kidneys. Whereas ER stress increased programmed cell death in NRK-52E cells, HDAC6 inhibition attenuated programmed cell death and was associated with induction of TFEB activity as evidenced by an increase in LAMP-1 transcript levels. Functionally, increased cell death of proximal tubule cells is known to contribute to the release of inflammatory cytokines, and fibrotic remodelling in the tubulointerstitium (Hodgkins and Schnaper, 2012), and the reduction of programmed cell death points to a potentially renoprotective role of HDAC6 inhibition and its associated increase in TFEB activation of the autophagy-lysosomal pathway. This finding is consistent with research that shows a bi-directional relationship between programmed cell death and autophagy. While autophagy increases as an initial protective step, chronic disease can initiate pro-death pathways that dampen the autophagic response and favour programmed cell death. For example, an increase an ER stress activates an intrinsic signalling pathway mediated by the B-cell lymphoma 2 (Bcl-2) family. As a consequence, Bcl-2 interacts with the autophagy protein beclin-1 and prevents the
formation of beclin-1 mediated autophagosome formation (Li et al., 2015). Furthermore, activated caspases in the apoptosis pathway cleave autophagy related proteins such as ATGs, further dampening the autophagic response (De Rechter et al., 2016). In contrast, increasing autophagy, as we have potentially done by increasing TFEB translocation, can also reduce pro-apoptotic pathways, placing greater dependence on cellular degradative processes before the cellular decision to undergo programmed cell death. For example, an increase in autophagy is accompanied by an increase in autophagy related genes such as Atg12 and Atg3. Atg12 conjugation with Atg3 increases Bcl-XL expression, a potent inhibitor of apoptosis (Tait et al., 2014). Therefore, increasing the autophagy-lysosomal pathway by way of increased TFEB activity may also increase autophagy mediated suppression of apoptosis. A limitation of the present study is our choice to focus on the ultimate downstream consequences of altered TFEB activity, namely, the accumulation of misfolded proteins, programmed cell death and renal decline. As a result, we have not defined whether and to what extent HDAC6 inhibition directly affects autophagic processes in a TFEB dependent manner. In-vivo, at least, it has been difficult to tease out given the temporal nature of autophagy in both its pro and anti-apoptotic effects.

Mechanistically, our in-vitro experiments point to a potential role of TFEB acetylation in regulating TFEB activity. However, de-phosphorylation of TFEB is the classically understood method of nuclear translocation and in this study, I did not assess the effect of HDAC6 inhibition on the enzymatic activity of phosphatases, such as calcineurin (Medina et al., 2015), that are involved in regulating de-phosphorylation and nuclear translocation of TFEB. Nonetheless, this study demonstrates that HDAC6 inhibition alters TFEB activity, a clinically relevant finding given the observation that TFEB is dysregulated and misfolded proteins accumulate in CKD. With the goal of assessing HDAC6 mediated regulation of TFEB as a potential avenue to reduce
the burden of CKD, in the next phase of this study, I assessed the effect of HDAC6 inhibition on renal structure and function in an experimental model of CKD, namely, the SNx rat.

6 **Tubastatin A is renoprotective in subtotally nephrectomized rats**

Consistent with our findings in-vitro, Tubastatin A enhanced the nuclear localization of TFEB in rat kidneys. While p62 labelled aggregates accumulated in renal tubules of vehicle treated SNx rats, Tubastatin A treatment prevented the accumulation of p62 in SNx rats. The increase in TFEB nuclear translocation and reduction in p62-labelled protein aggregates were associated with a reduction in tubule epithelial cell death in Tubastatin A treated SNx rats. Structurally, this was accompanied by a reduction in tubulointerstitial (but not glomerular) fibrosis and functionally this was associated with attenuation of urine protein excretion without significant improvements in GFR.

A key strength of this study’s design was that treatment was initiated after the onset of proteinuria, four weeks after renal ablation. This is clinically relevant because it mirrors a clinical scenario in which patients may present with proteinuria in the earlier stages of CKD (Regeniter et al., 2009). Following the initiation of Tubastatin A treatment in SNx rats after the fourth week, urine protein levels stabilized and did not significantly progress by the seventh week of study, suggestive of stabilization of kidney function over time. This attenuation of proteinuria was accompanied by a decrease in misfolded protein accumulation, a reduction in tubule epithelial programmed cell death and an attenuation of tubulointerstitial fibrosis. Although it is tempting to speculate that a
causal relationship exists between the decrease in misfolded protein accumulation and improvements in kidney structure and function under diseased conditions, it should be recognized that it has not been proven in the present study. In other diseases however, such as neurodegenerative diseases, a reduction in misfolded protein aggregates has been implicated in improved outcomes (Ciechanover and Kwon, 2015; Sarkar et al., 2007; Tanaka et al., 2004). Whether the same processes apply to the replicating tubule epithelial cells in CKD requires further study.

Interestingly, kidney weight was also reduced in SNx rats treated with Tubastatin A. The reasons for this are unclear. It could effect a change in cell size (hypertrophy) or cell number (hyperplasia). HDAC6 inhibition reduces tubule cell proliferation in polycystic kidney disease (Cebotaru et al., 2016). Whether the change in renal mass is attributable to anti-proliferative effects of HDAC6 inhibition in SNx rats remains unclear.

Importantly, although Tubastatin A appears to attenuate renal decline as measured by proteinuria, I did not see significant changes in GFR between vehicle and Tubastatin A treated SNx rats. This is perhaps unsurprising. While GFR decline begins to appear by stage 3 CKD, with further declines becoming evident as the disease progresses, the changes in GFR in the SNx model are largely governed by acute changes following subtotal nephrectomy, for which, reports have noted an immediate drop in GFR shortly after renal ablation surgery (Kaufman et al., 1974). This acute drop in GFR may be the driving force in the decline in GFR observed seven weeks after surgery. In contrast, proteinuria does not occur as an acute result of the surgery itself and is evidence of
progressive changes in the SNx kidney as a result of hyperfiltration, intraglomerular hypertension and subsequent proteinuria (Palatini, 2012).

Furthermore, in their work assessing the ACEi enalapril on glomerular injury in SNx rats, which today is main-stay therapy for CKD, Anderson and colleagues observed that ACE inhibition attenuated proteinuria 8 weeks after renal ablation without significant changes in GFR (Anderson et al., 1985). Therefore, the fact that Tubastatin A imparts renoprotective benefits on proteinuria and not in GFR does not negate the therapeutic potential of HDAC6 inhibition. While Tubastatin A has demonstrated comparable renoprotective benefits to current therapies for CKD, other HDAC6 inhibitors that are already being tested for safety in patient populations will likely see more success in transferability to the clinic. For example, whereas Tubastatin A has not progressed to clinical study, rocilinostat (ACY-1215) is another HDAC6 inhibitor (IC_{50}=5 nM) that is currently in phase I/II clinical trials for multiple myeloma (NCT01323751) and lymphoid malignancies (NCT02091063).

However, in considering the renoprotective effect of HDAC6 inhibition in CKD, and its regulation of TFEB, which is itself involved in autophagy, it is worth considering the alternative roles of HDAC6 in other steps of the autophagy-lysosomal pathway. HDAC6 plays multiple roles in promoting the autophagy-lysosomal pathway, which may, at first glance, be discordant with our observation of a renoprotective effect of its inhibition. As an aggresome organizer, HDAC6 serves as an adaptor protein between polyubiquitinated proteins and the dynein motor complex for microtubule retrograde transport to the peri-nuclear aggresome (Kawaguchi et al., 2003b; Kopito, 2000; Olzmann et al., 2008). However, work by Lee and colleagues draw a distinction
between quality control autophagy and nutrient-starvation autophagy. Whereas HDAC6 is important for cortactin mediated F-actin cytoskeletal remodelling for autophagosome lysosome fusion, this function is only apparent under basal, quality control autophagy. Under stressed conditions, HDAC6 appears dispensable for this role, and may instead, play an alternative, pathological role by dampening the cell’s ability to mount an autophagic response by sequestering TFEB in the cytoplasm.

Finally, it remains possible that the effects of HDAC6 inhibition on improving renal structure and function in SNx rats are multifactorial and not solely due to increased TFEB activity. Current established therapies that are known to improve renal outcomes (e.g. ACEi (Jafar et al., 2003) and sodium glucose cotransporter 2 (SGLT2) inhibition (Cherney et al., 2014) likely have multiple cell and organ effects. It seems likely therefore, that HDAC6 inhibitors may likewise have multiple sites of action. For example, HDAC6 inhibition improved renal function and decreased TGF-β (Choi et al., 2015b) and also prevented the development of polycystic kidney disease (Cebotaru et al., 2016). In line with these finding, the experiments detailed here confirm the renoprotective effect of HDAC6 inhibition and they identify TFEB acetylation as a novel means by which these effects may occur.
In conclusion, this study found that in human diabetic kidney tissue and SNx rats, mRNA levels of transcription factor EB (TFEB) were decreased. This was accompanied by an accumulation of p62-labelled protein aggregates in renal tubules. Given the apparent inability to contend with increasing amounts of protein aggregates in CKD, we set out to identify methods to increase activation of TFEB by facilitating its nuclear localization by way of HDAC6 inhibition. We found that inhibition of HDAC6 increased acetylation of TFEB, increased its nuclear translocation and reduced tubule epithelial cell death and this was associated with an attenuation of progressive proteinuria and reduced structural remodelling in SNx rats. The main findings of this study are summarized in Figure F. Collectively, this study highlights a regulatory relationship between HDAC6 and TFEB and it points to the therapeutic benefit of augmented quality control mechanisms in the treatment of CKD.

Figure F. HDAC6 inhibition facilitates transcription factor EB mediated clearance of misfolded protein in chronic kidney disease.
Chapter 7
Future Directions

My thesis aimed to evaluate a potential regulatory relationship between HDAC6 and TFEB in increasing the clearance of misfolded proteins in CKD. We demonstrated a dysregulation of TFEB in human diabetic kidney disease and in a model of advanced chronic kidney disease. Through in-vitro work, I found that HDAC6 inhibition led to hyperacetylation of TFEB and improved tubule epithelial cellular viability. In-vivo, HDAC6 inhibition induced TFEB nuclear translocation, increased clearance of misfolded proteins and attenuated renal functional and structural decline.

Beyond the limitations and future experiments already discussed, there are a number of additional directions to consider. Whereas a downregulation of TFEB is associated with advanced kidney disease in humans and SNx rats, we have not demonstrated a causal link between a downregulation in TFEB and the development of CKD. To gain more insight into the contribution of dysregulated TFEB in CKD, we could assess the effect of TFEB knockout on kidney function in a model of progressive renal disease such as the unilateral ureteral obstruction model (UUO) (Chevalier et al., 2009). Homozygous knockout of TFEB in mice is embryonically lethal (Steingrimsson et al., 1998) and this creates a limitation to the use of a conventional knockout mouse system. Instead, we could approach this problem in one of two ways. First, we could opt to study the progression of kidney disease following UUO in TFEB heterozygous mice, which, according to the International Mice Phenotyping Consortium (IMPC), develop normally with mild
skeletal defects and changes in body mass (http://www.mousephenotype.org/data/genes/MGI:103270). While this would provide some insight, the global deficiency of TFEB may impart effects on other organs. For example, enhancement of TFEB activity is protective against myocardial dysfunction (Unuma et al., 2013) which is itself, an independent contributor to CKD (Keith et al., 2004). As such, this would limit our ability to discern a causal relationship between downregulation of TFEB in diseased kidney tissue and changes in renal function. To address this issue, we could generate an inducible, kidney specific TFEB mouse model. To target TFEB knockout to renal epithelial cells, we could mate tamoxifen-inducible KspCad-CreER<sub>T2</sub> (Cre) mice (Lantinga-van Leeuwen et al., 2007) with TFEB floxed mice (http://www.informatics.jax.org/allele/allgenoviews/MGI:4431759).

In addition, having demonstrated increased TFEB activity through its nuclear localization, I am aware that this study is limited in its lack of direct demonstration of increased autophagy-lysosomal activity, beyond transcriptional changes in LAMP-1. To better characterize a temporal relationship between HDAC6 inhibition, TFEB translocation and autophagic activity, additional measures of autophagosome formation and lysosomal biogenesis could be assessed in future experiments. These assessments could include immunoblotting to assess changes in protein levels of proteins involved in autophagy, such as the conversion of microtubule associated protein 1A/1B light chain 3B (LC3), autophagy related proteins (ATGs) and beclin-1 (Mizushima et al., 2010) in NRK-52E cell treated with Tubastatin A in the presence or absence of TFEB knockdown with siRNA as well as RT-qPCR to assess multiple TFEB targets (Palmieri et al., 2011).
Next, given the multi-faceted role of HDAC6 in the autophagy pathway, through both its catalytic and non-catalytic function, it would be important to better characterize the effect of inhibition/deletion of HDAC6 on renal function and structure. In the present study, I compared TFEB transcript levels between humans with diabetic kidney disease and non-diabetic rats with CKD. In future studies, it would be useful to explore TFEB transcript levels and misfolded protein accumulation in a diabetic mouse model. Accordingly, to address both of these queries, we are currently in the process of breeding HDAC6-deficient mice with Akita diabetic mice that develop diabetes and subsequently, diabetic kidney disease due to a mutation in the insulin 2 (Ins2) gene (Gurley et al., 2010). In these experiments, we will survey TFEB mRNA and p62 protein levels and we will compare the effects of HDAC6 inhibition with Tubastatin A and HDAC6 knockdown in Akita diabetic mice. In addition to these in-vivo assessments, to gain mechanistic insight, one could turn to an in-vitro system to determine which of the two catalytic sites of HDAC6 (DD1 or DD2 or both) are responsible for mediating the acetylation of TFEB and its nuclear localization. DD1, DD2 and DD1/DD2 HDAC6 mutants have been developed (Ran et al., 2015) and we have recently received them in the lab. In future work, we will knockdown native HDAC6 with siRNA and transfect NRK-52E cells with these HDAC6 mutants to assess the consequences of DD1, DD2 and DD1/DD2 mutations on TFEB acetylation (immunoprecipitation) and nuclear localization (immunoblotting and immunofluorescence microscopy).

Finally, while HDAC6 inhibition serves a renoprotective role, we did not explore the potential of dual therapy with both renin-angiotensin blockade and HDAC6 inhibition. A future in-vivo study could assess the renoprotective effect of Tubastatin A and ACE inhibition or angiotensin II receptor blockade in a model of kidney disease reminiscent of human CKD, namely, the SNx rat model. From a clinical perspective, this is important because any new therapy is likely to be
applied to patients on top of standard of care, which is currently renin-angiotensin blockade (Jafar et al., 2003) and this study will enable us to assess whether any additional benefit can be gleaned by the addition of an HDAC6 inhibitor to current therapy.

Despite these limitations, this study is, to the best of my knowledge, the first to demonstrate that TFEB is diminished in CKD and the first to show a functional relationship between TFEB and HDAC6. Both TFEB and HDAC6 represent viable targets to explore in future studies aimed at slowing renal decline in CKD.


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Appendix

**10X Transfer Buffer**

- 144 g glycine
- 30.3 g Tris
- 1 L distilled water

Dilute to 1X by adding 100 mL of 10X Transfer Buffer to 900 mL of water. Then add 200 mL of methanol to make 20% v/v 1X Transfer Buffer.

**10X Running Buffer**

TG-SDS Buffer (Tris-Glycine-sodium dodecyl sulfate) 10X solution (BioBasics, Markham, ON, Canada). Add 100 mL of the 10X Running Buffer stock solution to 900 mL of distilled water to make 1X Running Buffer.

**10X TBS Buffer**

- 24.2 g Tris base
- 80 g NaCl

Adjust pH to 7.6 in 1 L of distilled water. Add 100 mL of 10X TBS stock solution to 900 mL of distilled water to make a 1X working solution. At 1000 µL of Tween 20 (BioShop, Burlington, ON, Canada) to the solution to make a 1X TBT-T working solution.

**5% Blocking Solution (for Immunoblot)**

- 1 g of skim milk powder (BioShop)
- 20 mL of TBS-T
Add reagents to a 50 mL conical tube. Vortex on high speed. Use 10 mL per nitrocellulose membrane.

**2% Blocking Solution (for Immunofluorescence)**

- 2 mg of bovine albumin serum (BSA) (Sigma-Aldrich)
- 10 mL of 1X phosphate buffer saline (PBS)

Add reagents to a 15 mL conical tube. Vortex on high speed. Use 10 mL per nitrocellulose membrane.

**Citric Acid Buffer**

- 41 mL (1M) sodium citrate
- 9 mL (1M) citric acid
- 500 µL (10N) NaOH
- 4950 mL of distilled water

Combine reagents and store at 4°C.

**Scott’s Tap Water**

- Sodium bicarbonate 8.75 g
- Magnesium sulphate 50.0 g
- Distilled water 2500 mL

**Homogenization Buffer**

- Sucrose 250mM 125 mL
- TrisHCl 10mM 5 mL
- EDTA 1mM 1 mL

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- Sodium orthovanadate 1mM 5 mL
- Sodium fluoride 1mM 0.5 mL
- Water 363 mL

Combine reagents and store at 4°C. Supplement with phosphatase inhibitor at a ratio of 1:1000 just prior to use.

5% FITC-inulin

- 100 mg FITC-inulin
- 2 mL 0.9% sodium chloride (NaCl) solution

1. Combine FITC-inulin and NaCl, slowly bring the solution to a boil.

2. Remove unbound FITC by filling solution into a cut-off dialysis membrane (1000 Da) (Spectrum Laboratories Inc., Rancho Dominguez, CA).

3. Submerge the filled dialysis membrane in 1 L of NaCl, under constant rotation for 24 hours at room temperature.

4. Sterilize the solution prior to injection by filtering solution through a 0.22 µm filter.