Mechanisms of Substrate Recognition by the Cul3-based E3 Ligase

Katia Graziella de Oliveira Rebola
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Mechanisms of Substrate Recognition by the Cul3-based E3 Ligase

by

Katia Graziella de Oliveira Rebola

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Biology

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ABSTRACT

Cul3-based E3 ligase is responsible for regulating a variety of cellular pathways, many of which are known to have profound effects on the proper function of multicellular organisms. Although progress over the past years has been truly impressive, our understanding of the mechanisms of E2 recruitment and selection by the BCR complex and all the roles that Cul3 plays on kidneys remains in its infancy. To explore these aspects, this dissertation aims to analyze the Cul3 complex using two different approaches: (1) We used the powerful tool of chimeric analysis to map the essential domain binding characteristics of Cul3 taking advantage of the fact that the well-characterized cullin family members exist with non-redundant functions. We hypothesized that besides the substrate recruitment role, Cul3 substrate selection subunits must also be involved in E2 selection since the E2 is responsible for determining the branching pattern of ubiquitin. From this analysis, we characterized a unique role for the substrate adaptor subunits. (2) We performed a quantitative proteomics analysis utilizing a newly created kidney cell line with Cul3 deleted to identify potential new degradation substrates in kidneys. We hypothesized that the phenotypic difference between Cul3 knockouts in kidneys and known Cul3 substrate knockouts in kidneys imply other important substrates exist. This analysis identified both known substrates, validating the study, and new and novel substrates that remain to be characterized.
DEDICATION

To my grandfather Valdeci Teixeira de Oliveira, who loved me from the depth of his heart.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

4HB – Four-helix bundle
AAD – Active adenylation domain
AD – Alzheimer’s disease
APPBP1 – Amyloid Precursor Protein-Binding Protein 1
ATP – Adenosine triphosphate
Aβ – β-amyloid
BRCA1 – Breast Cancer Type 1 Susceptibility Protein
BRMS1 – Breast Cancer Metastasis-Suppressor 1
BTB – Broad-Complex, Tramtrack and Bric a brac
c-CBL – Casitas B-lineage Lymphoma
CAND1 – Cullin-associated NEDD8-dissociated protein 1
CD8+ – Cluster of differentiation 8
CDK – Cyclin-dependent kinase
CP – Core particle
CRL – Cullin-RING ubiquitin ligases
SRS – Substrate-recognition subunit
CSN – COP9 signalosome
CSN5 – COP9 signalosome subunit 5
CUL1 – Cullin1
CUL2 – Cullin2
CUL3 – Cullin3
CUL4A – Cullin4A
CUL4B – Cullin4B
CUL5 – Cullin5
CUL7 – Cullin7
Cys – Cystine
DAXX – Death-associated protein 6
DCN1 – Defective in cullin neddylation protein 1
DCT – Distal convoluted tubule
DDB1 – DNA damage-binding protein 1
DNA – Deoxyribonucleic acid
DUB – Deubiquitinating enzyme
E1 – Ubiquitin-activating enzyme
E2 – Ubiquitin-conjugating enzyme
E3 – Ubiquitin-ligase
ERK1/2 - Extracellular signal-regulated protein kinases 1 and 2
FCCH – First catalytic cysteine half-domains
FHHt – Familial Hyperkalemia and Hypertension
GLI1 – Glioma-Associated Oncogene Homolog 1
GLI2 – Glioma-Associated Oncogene Homolog 2
Gly – Glycine
HECT – Homologous to the E6-AP Carboxyl Terminus
IAD – Inactive adenylation domain
IxkB – IkappaB kinase
JAB1 – Jun activating binding protein
kDA – Kilodalton
KEAP1 – Kelch-like ECH-associated protein 1
KLHL3 – Kelch Like Family Member 3
Lys – Lysine
LZTR1 – Leucine-zipper-like transcriptional regulator 1
MDM2 – Mouse double minute 2
MHC I – Major histocompatibility class I
MoeB – Molybdoopterin-synthase adenyllyl transferase
NAE – NEDD8-activating enzyme E1
NANOG – Homeobox protein NANOG
NEDD8 – Ubiquitin-like protein Nedd8
Nf-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells
NRF2 – Nuclear factor erythroid 2-related factor 2,
p53 – Tumor protein p53
PD – Parkinson’s disease
PHAII – Pseudohypaldosteronism type II
POZ – Pox virus and Zinc finger
PTM – Post-translational modification
RAS – Rat sarcoma
Rb – Retinoblastoma protein
RBR – RING-between RING-RING
RBX1 – RING Box Protein 1
RBX2 – RING Box Protein 2
RING – Really Interesting New Gene
RNA – Ribonucleic acid
ROC1 – Regulator of Cullins-1
ROS – Reactive oxygen species
RP – Regulatory particle
Rpn – Proteasome non-ATPase regulatory subunit
SCCH – Second catalytic cysteine half-domains
SKP1 – S-phase kinase-associated protein 1
SOCS – Suppressor of cytokine signaling
SPOP – Speckle Type BTB/POZ Protein
ThiF – Sulfur carrier protein ThiS adenylyl transferase
TKI – Tyrosine kinase inhibitor
TRIM40 – Tripartite motif containing 40
Ub – Ubiquitin
UBA1 – Ubiquitin Like Modifier Activating Enzyme 1
UBA3 – NEDD8-activating enzyme E1 catalytic subunit
UBA6 – Ubiquitin Like Modifier Activating Enzyme 6
UBB – Ubiquitin B
UBC – Ubiquitin conjugation domain
UBE2F – Ubiquitin Conjugating Enzyme E2 F
UBE2M – Ubiquitin Conjugating Enzyme E2 M
UBE2Z – Ubiquitin conjugating Enzyme E2 Z
UFD – Ubiquitin-fold domain
**UPS** – Ubiquitin-dependent proteasome system

**USE1** – Unconventional SNARE in the ER 1

**USP** – Ubiquitin-specific protease

**WDXR** – WD40-Containing motif

**WNK1** – With No Lysine 1

**WNK4** – With No Lysine 4
GLOSSARY

**BTB:** A domain found in proteins that function as Cul3 substrate adaptors. The BTB domain binds to Cul3.

**Cre:** Cre recombinase is an enzyme that can be used in a conditional knockout mouse model to recombine the DNA that is surrounded by two LoxP sites (floxed).

**Ctb73:** A putative substrate adaptor for Cul3, Ctb73 contains an N-terminus BTB domain, a central BACK domain, and a C-terminus PHR domain.

**Cullin-based E3 ligases:** They are multi-subunit E3 ubiquitin ligases which use a specific cullin as a central scaffold. All the cullin complexes use their C-terminus to recruit a RING-Box protein (Rbx1 or Rbx2), required for the interaction with an E2, and their N-terminus to interact with the substrate adaptor subunit.

**Cul1:** One of two E3 ligases responsible for cyclin E ubiquitination. Cul1 uses substrate adaptors with two subunits: The Skp1 linker protein and an F-box protein.

**Cul3 Δ403-459:** A Cul3 mutation that results in the skipping of exon 9 of the Cul3 protein. This mutation causes FHHt in humans.

**Cul3:** An E3 ubiquitin ligase that targets many substrates, including cyclin E, for ubiquitination. Cul3 uses BTB-domain containing proteins as substrate adaptors.

**Cul3Δ51-67:** This Cul3 mutant lacks the region near its N-terminus that is responsible for binding BTB proteins (substrate adaptors).

**Cyclin E:** Cyclin E controls the G₁/S transition in the cell cycle by binding and activating Cdk2. Cyclin E/Cdk2 complexes phosphorylate themselves in addition to many other substrates including the Rb protein. Eukaryotes contain two cyclin E proteins, cyclin E1 and cyclin E2, which are products of two different genes.
**E1 ubiquitin activating enzyme:** E1 enzymes are responsible for activating the C-terminus Gly-Gly motif on ubiquitin and preparing it for attachment to a substrate. Activation of ubiquitin by an E1 occurs via an ATP-dependent reaction.

**E2 ubiquitin conjugating enzyme:** E2 enzymes work with E3 ubiquitin ligases in order to transfer ubiquitin to substrates.

**E3 ubiquitin ligase:** E3 ligases provide substrate specificity to the ubiquitin system. Cullin E3s work with an E2 to facilitate ubiquitin attachment. HECT E3 ligases and cullin ligases represent two classes of E3s.

**F-box:** Cul1 utilizes substrate adaptors, such as Fbxw7, that contain F-box domains to recognize substrates.

**FAZF:** A BTB protein that also contains a zinc-finger domain. FAZF binds LRR5 (FMOD)

**Fbxw7:** An F-box protein that functions as a substrate adaptor for Cul1. Fbxw7 is involved in Cul1-mediated ubiquitination of cyclin E. xiii

**Keap1:** A prominent member of the BTB-BACK-Kelch family of proteins, Keap1 targets the transcription factor Nrf2 for degradation.

**Kelch domain:** A common substrate-recognition domain that is found in many BTB domain-containing proteins. Some notable members of this family are Keap1, which regulates Nrf2, and Klhl3, which is involved in blood pressure regulation.

**Klhl3:** A BTB-BACK-Kelch protein that has been shown to cause FHHt in humans when mutated.

**MATH domain:** A secondary domain that is found in the Cul3 substrate adaptor SPOP.
**MG132**: A proteasome inhibitor that when added to cultured cells, results in increased stability of some ubiquitin-proteasome system substrates.

**Nedd8**: Nedd8 is a ubiquitin-like molecule involved in Cul3 function. Cul3 is modified by Nedd8 on K712.

**Nrf2**: Nrf2 is a transcription factor involved in the stress-response. Nrf2 is a substrate of the Keap1 BTB-Kelch protein and Cul3.

**PLZF**: A zinc-finger domain containing BTB protein. PLZF is closely related to FAZF.

**Rb**: The retinoblastoma protein plays a crucial role in cell cycle progression, as it is responsible for binding and sequestering the E2 factor (E2F) family of transcription factors, which are believed to play a crucial role in cell division control. Sufficient phosphorylation of Rb by cyclin/Cdk pairs results in the release of E2F, allowing for transcription of S-phase associated genes to begin.

**Rbx1**: The “ring-finger” protein, which interacts with cullin ubiquitin E3 ligases near their C-termini. Rbx1 is believed to play a role in E2 binding.

**RhoA**: RhoA is a small GTPase that regulates many processes, including the actin cytoskeleton.

**RhoBTB3**: A member of the RhoBTB protein family that binds cyclin E.

**Skp1**: Skp1 forms part of Cul1-based complexes as it is responsible for linking Cul1 with an F-box protein.

**SPOP**: Also called Ctb75, SPOP is a Cul3 substrate adaptor (BTB protein).

**Substrate adaptor subunit**: Substrate adaptors are proteins that bind to cullin-based E3 ligases in order to recognize specific substrates for ubiquitination. Each
cullin ligase can associate with many different substrate adaptors, allowing each cullin to have many substrates.

**UbE2E1**: UbE2E1 is an E2 ubiquitin conjugating enzyme, which forms degradative (K48) ubiquitin linkages. UbE2E1 binds RhoBTB3.

**Ubiquitin**: Ubiquitin is a small protein that gets attached to target proteins, often resulting in their degradation.

**WNK1**: With no lysine (K) 1: A kinase involved in NCC regulation. WNK1 can bind and be ubiquitinated by Klhl3.

**WNK4**: With no lysine (K) 4: A kinase involved in NCC regulation. WNK4 interacts with and can be ubiquitinated by Klhl3.

**Zinc Finger domain**: A DNA-binding domain that is present in some BTB proteins, for example PLZF and FAZF.
CHAPTER 1

Background and Significance
INTRODUCTION

This dissertation focuses on the structure and function of a complex that modifies proteins by adding ubiquitin to them. This small protein is added in a multitude of complex ways, which then results in the modified protein being recognizable by many other proteins. The nature of the ubiquitin modification thus determines the fate of the protein. Our lab has been interested in a particular complex that accomplishes this called the Cul3 complex or BCR (BTB-Cul3-Rbx1). The entities that are capable of identifying and then directing the location of ubiquitination are called E3 ligases. In this portion of my dissertation, I will familiarize you with the process of ubiquitination, identify the major players, introduce what is known about Cul3 and develop some models that provide the framework for the testable hypotheses presented in subsequent chapters.

UBIQUITINATION

Post-translational modifications (PTMs) play an essential role in nearly all biological processes. These modifications provide versatile mechanisms for regulating a vast range of cellular functions through the covalent attachment of a functional group to a specific cellular target (Beck-Sickinger and Mörl, 2006).

Among several different PTMs, ubiquitination has emerged as the second most common protein modificatory process, behind only phosphorylation (Suresh et al., 2016). This process consists of the attachment of a small protein (76 amino acids) called ubiquitin to substrates and is mediated by the sequential action of three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme
(E2), and a ubiquitin-ligating enzyme (E3). Like most covalent modifications, ubiquitination is chemically stable and can only be removed by the action of proteases specialized in isopeptide-processing called deubiquitinating enzymes (DUBs; Hershko & Ciechanover, 1998). Together, these enzymes generate a complex signaling code, which targets the modified substrates for different pathways depending on the ubiquitin branching architecture on the ubiquitin they are modified with (Varshavsky, 2012). Protein degradation, endocytosis, traffic, autophagy, cell cycle progression, DNA stability, transcription, and translation are some of the cellular activities controlled by ubiquitination (Hershko et al., 1983; Finley, 2009).

**BIOLOGICAL SIGNIFICANCE OF THE UBIQUITINATION SYSTEM**

Ubiquitination plays a vital role in regulating proteins on the cellular level. Consequently, aberrations in the ubiquitination process can lead to several human disorders, including cardiovascular diseases, cancers, inflammatory and autoimmune disorders, neurodegenerative diseases, and developmental disabilities (Popovic et al., 2014). In the following sections, I will discuss conditions caused directly by genetic defects in components of the ubiquitin system or whose progression is modulated by the ubiquitin interaction network.

*Molecular mechanisms of ubiquitination dysregulation in cancers*

Ubiquitin modifications regulate multiple growth-signaling pathways in the cell, ranging from DNA replication and repair to chromosomal separation and
cytokinesis (Devoy et al., 2005). Given these central roles, it is not surprising that dysregulation in the ubiquitin signaling process plays a critical role in malignant tumor development (Senft et al., 2017; Deshaies, 2014). In fact, many critical cellular targets have been implicated in carcinogenesis and cancer cell survival, including Cyclin-dependent kinase (CDK)-cyclin complexes, retinoblastoma protein (Rb), and tumor suppressor p53 (Chen et al., 2004; Kalejta and Shenk, 2002; Pagano et al., 1995).

CDK-cyclin complexes are directly involved in the cell cycle progression, and disruptions in this process usually lead to genomic instability and uncontrolled cell growth, which are common characteristics of cancer cells (Ding et al., 2020). Proteins p53 and Rb belong to a complex protein network responsible for regulating the cellular response to stress and DNA damage (Love et al., 2013). However, mutations generally lead to conformational changes in p53 and Rb structures, which prevent them from being ubiquitinated and subsequently targeted to degradation by the proteasome. Consequently, the feedback loop that controls the p53 and Rb cellular levels is disrupted; they accumulate at extremely high levels in the cells and become unable to suppress further tumor development (Inoue et al., 2012).

Another important tumor suppressor regulated by ubiquitin modification is the cyclin-dependent kinase (Cdk) inhibitor p27 (Alessandrini et al., 1997). p27 acts as a negative regulator of the cell cycle by inhibiting the activity of cyclin/cdk complexes during G0 and G1 phases of the cell cycle. Degradation of p27 is required to transition from G1 to S phase and occurs through ubiquitination and
subsequent degradation by the proteasome (Montagnoli et al., 1999; Tsvetkov et al., 1999). Several studies have shown that the enhanced degradation of p27 is observed in many types of aggressive human carcinomas (Vlach et al., 1997; Bloom & Pagano, 2007), and mutations in the p27 gene have been linked to higher tumor grade and poor prognosis (Slingerland & Pagano, 2000).

Molecular mechanisms of ubiquitination dysregulation in inflammatory and autoimmune diseases

Multiple lines of evidence have indicated that the ubiquitin modifications also play a role in the development of immune and inflammatory responses, including in the major histocompatibility (MHC) class I antigen processing and NF-κB signaling pathway activation (Marfella et al., 2006; Wang and Maldonado, 2006; Hu and Sun., 2016). MHC class I molecules are usually expressed on the cell surface of nucleated cells and present peptide fragments derived from intracellular proteins processed by the proteasome (Cascio et al., 2001). In addition, ubiquitin modifications have been shown to regulate macrophages activity and CD8+ T lymphocyte metabolism (Cascio et al., 2001; Widjaja et al., 2017). Although ubiquitination has a unique value for the body's immune response, the most critical link between the ubiquitin system and the immune system is related to NF-κB. NF-κB is a master regulator of DNA transcription, cell survival, cytokine production, and response to infection. In unstimulated cells, NF-κB is actively inhibited when bound to IκB. Upon stimulation, the phosphorylated IκB is ubiquitinated and degraded by the proteasome. This process allows NF-kB to translocate to the
nucleus, where it mediates the expression of several genes involved in inflammation (Wang and Maldonado, 2006; Wu et al., 2018). Therefore, any dysregulation in the ubiquitination process can have drastic effects on the body’s defense mechanisms.

**Molecular mechanisms of ubiquitination dysregulation in neurodegenerative disorders**

A growing body of evidence has also associated some chronic neurodegenerative diseases with increased ubiquitination levels in different neural structures, such as in the brainstem Lewy bodies of Parkinson's disease and the neurofibrillary tangles of Alzheimer's disease (Schwartz and Ciechanover, 2009). Parkinson's disease (PD) is a disorder that affects predominately dopaminergic neurons in a specific area of the brain called substantia nigra. The cause remains largely unknown, but researchers are currently exploring ways to identify biomarkers that can lead to a better understanding of the disease's progress. A series of physiological and genetic analyses revealed that abnormal functions of alpha-synuclein impaired the activity of the ubiquitination system, and accumulation of ubiquitinated proteins in Lewy bodies were observed in the brain of patients with Parkinson's (Zheng et al., 2016).

In the case of Alzheimer's disease, the most common symptom is the progressive degeneration of the neural system, which involves the accumulation and aggregation of neurotoxic proteins, such as β-amyloid (Aβ), hyperphosphorylated tau, and ubiquitinated proteins in vulnerable areas of the
brain, such as the hippocampus and cortex. Similar to PD, the ubiquitination system has been the subject of a recent focus in Alzheimer's disease pathogenesis, especially after studies have demonstrated that there is a reduction in proteasomal activity in AD brains and that the UBB+1, a genetic variant of ubiquitin that is unable to tag protein substrates covalently, is involved in neuronal degeneration through neuritic beading and mitochondrial stress (George et al., 2018). These accumulated pieces of evidence in the two most common neurodegenerative disorders indicate that dysfunctions in the ubiquitination system are a crucial factor in initiating and aggravating their pathogenesis.

**Molecular mechanisms of ubiquitination dysregulation in developmental disabilities**

Autism spectrum disorders (ASDs) are multifaceted conditions characterized by impairments in cognition, communication, and behavior (CDC, 2019). *De novo* mutations in the Cullin3-RING E3 ligase (Cul3) gene were recently found in distinct cohorts of autism patients by large-scale unbiased genetic analysis, making Cul3 one of the top-ranking high-risk autism factors (Kong et al., 2012). In addition, Cul3 was one of the 107 risk genes identified in a genome-wide association study (GWAS) of a large population of autism cases (De Rubeis and Bagni, 2011). Cul3 has also been associated with schizophrenia (SCZ) in a multi-stage SCZ GWAS spanning 108 conservatively defined loci, and its mutations overlap with those in ASDs (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Although Cul3 is abundantly expressed in the brain (Dong et al., 2020), there is little information about its binding proteins or the
pathways in which this E3 ligase might be involved. Currently, there are no biomarkers to identify ASDs, and the diagnosis is primarily based on clinical evaluation. This is problematic because such a lack of specificity presents a challenge for nosology and the development of more precise therapies. To address this problem, several ongoing studies are attempting to identify potential biomarkers, such as Cul3 or other components of the ubiquitin-dependent proteasome system, that could help with the diagnosis, discrimination, and prognosis of different ASDs (Arons et al., 2013; Srivastava and Schwartz, 2014; Faraone et al., 2014; Hallak et al., 2015).

Considering the broad functional spectrum of the ubiquitination network and the large number of proteins that participate in it, further disease-causing mutations within the ubiquitination system await discovery. Undoubtedly, technical advances in detecting ubiquitinated proteins and identifying their respective receptors will stimulate progress in this research area.

THE COMPONENTS OF THE UBIQUITINATION SYSTEM

The ubiquitination system is directly or indirectly involved in all aspects of metabolic networks linked to either normal or pathologic pathways. This complex cellular system encompasses not only the enzymes required for catalyzing the attachment of ubiquitin to substrates but also the proteins that bind to ubiquitinated proteins and lead them to their final fate. It also includes activities that remove ubiquitin independent of, or in concert with, proteolysis of the substrate, either by
the proteasome or proteases in the vacuole. Below, the main components of the ubiquitination network are described, with special attention given to the current data regarding the modulation of the different parts of this complex cellular system (Figure 1.1).

Ubiquitin (originally, ubiquitous immunopoietic polypeptide)

Considered the core component of the ubiquitination process, ubiquitin is a 76 amino acid-long protein with a molecular weight of approximately 8.5kDa (Ciechanover et al., 1978). It was discovered (as a free protein) in 1975 by Gideon Goldstein and named after the Latin word *ubique*, which means "everywhere" because it was found *ubiquitously* in all eukaryotic cells (Goldstein et al., 1975). At first, it was assumed that ubiquitin was only present in Eukaryotes (Özkaynak et al., 1984; Dworkin-Rastl et al. 1984; Bond and Schlesinger, 1987), but recent studies demonstrated that Archaea and Bacteria have ubiquitin-like systems as well (Maupin-Furlow, 2013; Pisano et al., 2018). Four different genes (UBB, UBC, RPS27, and UBA52) encode ubiquitin in mammals, in which genes UBB and UBC encode linear fusions of 3 and 9 ubiquitin peptides, respectively, whereas RPS27A and UBA52 encode ubiquitin as an in-frame fusion to a small and large ribosomal protein, respectively (Özkaynak et al., 1984; Finley et al., 1989; Redman & Rechsteiner, 1989).

The structure and basic functions of ubiquitin, as well as the components of the ubiquitination machinery, including the identification of E1, E2, and E3 enzymes (Figure 1.2; Vijay-kumar et al., 1987; Ciehanover et al., 1982; Hershko
et al., 1983), were elucidated throughout the late 1970s and 1980s by Aaron Ciechanover, Avram Hershko, and Irwin Rose for which the Nobel Prize in Chemistry was awarded in 2004 (Nobel Prize in Chemistry, 2004). These discoveries were followed by a set of studies led by Alexander Varshavsky’s research group, which revealed the biology of the ubiquitin system, including its importance for protein degradation in vivo, its physiological roles (in the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses), the origin of its selectivity (specific degradation signals in defective and short-lived proteins), and its critical mechanistic attributes, such as the poly-ubiquitin chain assembly and the selectivity of protein degradation (Ciechanover et al., 1984; Finley et al., 1984; Özkaynak et al., 1984; Bachmair et al., 1986; Jentsch et al., 1987; Goebel et al., 1988; Bachmair & Varshavsky, 1989; Chau et al., 1989; Wünning et al., 1989; Bartel et al., 1990; Johnson et al., 1990).

**Ubiquitin-activating enzyme (E1)**

The ubiquitination cascade initiates with the activation of ubiquitin by a ubiquitin-activating enzyme (E1) in an adenosine triphosphate-dependent manner. In vertebrates, the group of ubiquitin-activating enzymes is represented by two members of the E1 family, Uba1 and Uba6. Uba1 and Uba6 demonstrate distinct preferences for E2 charging in vitro and have a relative abundance ratio of >10:1 (McGrath et al., 1991; Schulman and Wade Harper, 2009; Clague et al., 2015). They are ubiquitously expressed in different organisms, ranging from humans to zebrafish. Several studies demonstrate that the expression of Uba1 is essential
and, although the Uba6 has also been shown to be capable of activating ubiquitin, the exceptionally high expression of Uba1 suggests that the ubiquitin pathway does not rely on activation by Uba6. In fact, Uba6 is only required for embryonic development and is uniquely responsible for transferring ubiquitin to UbE2Z (USE1), a Uba6-specific E2 (Jin et al., 2007).

Uba1 is a multidomain enzyme and by far the best-understood example of E1 in humans (Handley et al., 1991). Each of its domains plays a distinct functional role: the active and inactive adenylation domains (AAD and IAD) are responsible for adenylyating the C-terminus of ubiquitin (Lake et al., 2001; Lois and Lima, 2005); the Cys domain (split into first and second catalytic cysteine half domains, SCCH and FCCH) harbors the catalytic cysteine for thioester bond formation with ubiquitin (Szczepanowski et al., 2005); and the ubiquitin fold domain (UFD) is the one involved in molecular recognition of E2s (Huang et al., 2004; Huang et al., 2007). Due to the variety of cellular functions in which Uba1 is involved, mutations or a complete loss of the gene have been demonstrated to be lethal in several organisms (Yang et al., 2007; Groen and Gillingwater, 2015; McGrath et al., 1991).

*Ubiquitin-conjugating enzyme (E2)*

The second step of the ubiquitination cascade is coordinated by a group of proteins known as ubiquitin-conjugating enzymes (E2s). They are essential in the ubiquitination system as they regulate both the topology of the poly-ubiquitin chains and the processivity of the polyubiquitination reactions (Valimberti et al., 2015). Some E2s are extraordinarily flexible and accommodate a variety of
substrates, which allows for a multitude of distinct ubiquitination events and the generation of diverse substrate-ubiquitin chains. On the other hand, some E2s are more rigid and incapable of adding ubiquitin to anything other than ubiquitin itself. This specific subset is exclusively dedicated to extending chains with specific linkage types (Durfee et al., 2008; Clague et al., 2015; Eletr et al., 2005).

In the human genome, there are 35 E2s exclusively dedicated to ubiquitin conjugation. They are grouped according to the presence of appendages either N- or C-terminus to the catalytic domain (Classes II and III, respectively), at neither (Class I), or at both ends (Class IV). They all share a ~150-amino acid conserved catalytic core domain, known as the ubiquitin conjugation (UBC) domain, which is the minimal sufficient unit for the E2 enzymatic activity (Figure 1.3). This catalytic core domain contains the catalytic cysteine responsible for forming the thioester bond with ubiquitin. The structures of over 32 human E2s (full-length or UBC domain) have been solved, and the topologies of most are consistent with this canonical fold (Stewart et al., 2016).

**Ubiquitin-ligase (E3)**

The last step of the ubiquitination cascade is accomplished by ubiquitin-ligases (E3s). They are the most critical components of the ubiquitination system because they are the ones that confer specificity to the process through the recognition of target substrates. Together, they comprise the largest and most heterogeneous class in the ubiquitination cascade, with 60–100 putative E3s in yeast and over 1000 in humans (Berndsen and Wolberger, 2014). They are
subdivided into three main groups that vary according to the presence of specific domains and the mechanisms of ubiquitin transfer to the substrates (Figure 1.4; Morreale and Walden, 2016).

The most abundant group is the RING-type E3 ligase, which is characterized by the presence of a zinc-binding domain known as RING or by a U-box fold catalytic domain. The U-box fold catalytic domain adopts a similar conformation as the RING domain but does not contain zinc (Zheng and Shabek, 2017). RING-type E3s function as a scaffold by orienting the ubiquitin-charged E2 and mediating the direct transfer of ubiquitin to the target substrate. They are known for their strong tendency to form homodimers and heterodimers, in which only the homodimeric RINGs can functionally interact with two E2s (one per each monomer) (Deshaiés and Joazeiro, 2009; Morreale and Walden, 2016). Like the E1s and E2s, several studies have shown that mutations of RING-type E3s, or dysregulation of their activity, are associated with an array of human disorders. One well-known example is the development of familial breast and ovarian cancers when BRCA1, an E3 that plays a critical role in DNA repair, is mutated (Metzger et al., 2014). Among all the RING-type E3 ligases, cullin-RING ubiquitin ligases (CRLs) comprise the largest known class. Characterized as multisubunit complexes, composed of a cullin, a substrate-recognition subunit (SRS), and a Rbx protein, they are implicated in the regulation of a diverse array of eukaryotic functions, such as cell cycle control, hypoxia signaling, development, and response to DNA damage (Bosu & Kipreos, 2008).
The second-largest group of ubiquitin-ligases is known as Homologous to the E6-AP Carboxyl Terminus (HECT)-type E3 ligases which, as implied by the name, are characterized by the presence of the HECT domain. Structurally, these enzymes are composed of a bulky N-terminus lobe (N-lobe) that contains the E2 binding domain and a C-terminus lobe (C-lobe) that carries the catalytic cysteine (Huibregtse et al., 1995). They usually catalyze the ubiquitin transfer to the substrate through a two-step reaction. First, ubiquitin is transferred from the E2 to a catalytic cysteine on the E3 and then from the E3 to the substrate (Weber et al., 2019). Like RING E3s, they regulate a wide range of cellular processes and are involved in many human disorders, such as malignant tumorigenesis (Wolyniec et al., 2012).

The last and recently described E3 group is composed of ligases that contain an RBR domain. Similar to HECT, they catalyze ubiquitin transfer through a two-steps reaction that initiates with the transfer of ubiquitin to a catalytic cysteine located in the C-terminus domain of the E3 and ends with the ubiquitin being transferred to the target substrate (Spratt et al., 2014). In addition, this unique family of E3 ligases includes parkin, a ligase that plays an essential role in the cellular stress response, whose dysfunction is linked to the pathogenesis of early-onset Parkinson's disease (Riley et al., 2013).

*Deubiquitinating enzyme (also known as Ubiquitin-specific proteases)*

The vast majority of cellular proteins are ubiquitinated during their lifetime (Peng et al., 2003; Wagner et al., 2011; Kim et al., 2011; Swatek & Komander,
and, in order to maintain the cell homeostasis, ubiquitin must be recycled once the substrate has been committed to a cellular pathway. This recycling process is performed by a class of enzymes known as deubiquitinating enzymes (DUBs), which are modular enzymes that contain catalytic domains and additional ubiquitin-binding domains, and several protein-protein interactions domains. These enzymes regulate ubiquitin modifications on proteins in different manners, ranging from removing ubiquitin from modified proteins, recycling ubiquitin attached to inappropriate targets, disassembling poly-ubiquitin chains, and processing proteins before their degradation by the proteasome (Nijman et al., 2005; Sippl et al., 2011).

**26S proteasome**

Despite the variety of possible cellular outcomes, a vast range of polyubiquitinated proteins is targeted to the 26S proteasome for degradation (Sadowski and Sarcevic, 2010). The 26S proteasome is considered the most critical non-lysosomal protease in Eukaryotes and is responsible for the degradation of numerous regulatory proteins in addition to damaged or misfolded polypeptides (Peters, 1994; Livneh et al., 2016). This protease is found in both the cytoplasm and the nucleus of all eukaryotic cells and is composed of two subcomplexes, a 750 kDa proteolytic core particle (CP; also known as the 20S subunit) and a 900 kDa regulatory particle (RP; also known as the 19S subunit). The regulatory particle (RP) coordinates the degradation process by recognizing the polyubiquitinated substrates, unfolding, deubiquitinating, and translocating
them into the interior of the core particle (CP), where they are degraded to oligopeptides (Tanaka, 2009).

In summary, the ubiquitination system is critical for the constitutive turnover of proteins in the nucleus and cytoplasm, as well as for the activation of proteins involved in virtually every cellular process, including cell cycle progression, transcriptional regulation, genome integrity, apoptosis, neuronal plasticity, and immune responses. Much has been learned about its components in the last five decades, but the mechanisms that regulate each element within this system have yet to be deciphered.

The most critical event in the entire process is the substrate recognition step, which is accomplished by the E3 ligase family. Our laboratory has been a significant player in disseminating the mechanisms of one E3 ligase called Cul3, which is a member of the cullin family of RING-type E3s. Thus, the work described in this dissertation focuses on the mechanisms that control the Cullin3-based E3 ligase (Cul3). To enlighten the readers about what is known about the cullin family of E3 ligases, a discussion of what is known about them is presented below.

CULLIN-RING UBIQUITIN LIGASES (CRLs)

Cullin-RING ubiquitin ligases comprise the largest known class of E3 ligases (Sarikas et al., 2011). The human genome encodes seven different cullins (Cul1, 2, 3, 4A, 4B, 5, and 7) that, collectively, mediate a vast number of cellular processes, including transcription, signal transduction, development, and multiple
aspects of the cell cycle (Kleiger and Deshaies, 2016). CRLs are multisubunit complexes composed of a cullin, a RING H2 finger protein, a substrate-recognition subunit (SRS), and an adaptor subunit that links the SRS to the complex (Figure 1.5). Cullin’s C-terminus domain tightly associates with the RING-H2-domain proteins Rbx1 or Rbx2, which bind the Ubiquitin-conjugating enzymes E2s. The N-terminus domain interacts with a specific set of SRS or adaptor subunits (Kamura, 1999). Three distinct types of adaptor subunits have been described: F-box, SOCS/BC-box, and DDB1. F-box binds to the SRS Skp1, which binds to Cul1 and Cul7 (Bosu and Kipreos, 2008). SOCS/BC-box binds to the SRS elongin BC, which binds to Cul2 and Cul5. Finally, DDB1 binds to an SRS that contains WD-repeats of a subclass called WDXR, which, in turn, binds to Cul4.

In Cul3, substrates can bind to the Cullin directly (Davidge et al., 2019) or indirectly through a single polypeptide that binds simultaneously to Cul3 through an N-terminus BTB-domain and to the substrate through a domain on the N- or C-terminus (Nguyen et al., 2017; Hill et al., 2019). The CRL complexes are generally inactive, but the covalent attachment of the ubiquitin-like protein Nedd8 causes a conformational change that switches them on (Duda et al., 2011). Together, cullin scaffold enzymes have an enormous impact on eukaryotic cell biology, and mutations in individual cullins have been associated with multiple diseases (Kleiger and Deshaies, 2016).
Nedd8

CRLs are activated through a process known as neddylation, in which the ubiquitin-like protein Nedd8 is covalently attached to a conserved lysine residue near the Cullin's C-terminus domain (Bosu and Kipreos, 2008). This post-translational modification plays an essential role in their activities because it can directly affect their stability, subcellular localization, conformation, and function (Duda et al., 2011). The peptide NEDD8 is structurally similar to ubiquitin and is also covalently linked to target proteins through a sequential action of its own set of E1, E2, and E3 enzymes. In mammalian cells, there is a single Nedd8 E1 (NAE), two E2s (UbE2M, also known as Ubc12, and UbE2F), and approximately twelve E3s. NAE resembles a standard ubiquitin-activating enzyme (E1) and consists of a catalytic subunit (Uba3/Naeβ) and a regulatory subunit (Nae1/Appbp1). Multiple data sources have shown that UbE2M preferentially promotes neddylation of Rbx1-associated cullins (Cul1, 2, 3, 4A, and 4B), whereas UbE2F promotes neddylation of Rbx2-associated cullins (Cul5; Zheng et al., 2021; Li et al., 2019).

Similar to CLRs, the vast majority of Nedd18 E3 ligases contain a RING domain, such as Rbx1, Rbx2, c-Cbl, Mdm2, and Trim40 (Kamura, 1999; Zuo et al., 2013; Noguchi et al., 2011; Xirodimas et al., 2004). Dcn1 enzyme is also involved in Nedd8-CRL conjugation, but unlike other Nedd8 E3s, it does not contain a RING domain for its catalytic activity (Kurz et al., 2008). Despite its complexity, neddylation plays an essential role in the ubiquitination cascade because it increases the affinity of the ubiquitin-charged E2 enzyme to the E3 ligase (Kawakami et al., 2001) and triggers structural changes that boost the transference.
of ubiquitin to the substrate (Duda et al., 2008). In addition, NEDD8 can also counteract the association of the CLRs with Cand1 (Liu et al., 2002). Cand1 is a cullin inhibitor that prevents the binding of Cullin to its substrate-specific subunits and blocks the lysine residue that becomes neddylated in the active complex (Goldenberg et al., 2004; Zheng et al., 2002).

**COP9 Signalosome**

Deneddylation, a process in which Nedd8 conjugates are removed from Cullins, is mediated by the isopeptidase activity of the metalloprotease CSN5/Jab1 subunit of the COP9 signalosome. This process is physiologically necessary because it regulates in a coordinated manner the activity of the CLRs, coordinates the exchange of their adaptor subunits, and prevents their auto-ubiquitination (Qin et al., 2020; Lingaraju et al., 2014; Cope and Deshaies, 2003). The COP9 signalosome is composed of eight highly conserved subunits, Csn1–Csn8. The homology of the COP9 signalosome to the eight subunits of the 19S proteasome lid complex and the three subunits of the eIF3 translation initiation factor complex suggest a common origin for these three protein complexes. These subunits can be found encoded in the genomes of many diverse organisms, including humans, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the mustard weed *Arabidopsis thaliana*, the fission yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae* (Bosu and Kipreos, 2008). Several studies have shown that COP9 signalosome is not just implicated in deneddylation (Wolf et al., 2003). In plants, COP9 signalosome plays a major in
growth, development, and defense against pathogens, while in yeast, it is involved in different mating pathways (Schwechheimer et al., 2000), and in yeast, it regulates different mating pathways (Maytal-Kivity et al., 2002). Studies in human cells showed that the COP9 signalosome also plays a vital role in signal transductions and repair of double-strand breaks by getting recruited to double-strand break sites in a neddylation-dependent manner (Hannß and Dubiel, 2009; Meir et al., 2015).

**BTB-CUL3-RBX1 (BCR) COMPLEX**

Cul3, a 768 amino acid-long protein, is a highly conserved CULLIN family member found in all eukaryotes. This E3 ligase is the core component of a complex known as BTB-Cul3-Rbx1 (BCR), which comprises the RING finger protein Rbx1, the Cul3 scaffold, and a Bric-a-brac/Tramtrack/Broad complex (BTB) protein. In these complexes, the BTB domain-containing protein is responsible for bridging Cul3 to the substrate in a single polypeptide, while Skp1/F-box or ElonginC/SOCS heterodimers fulfill this function in the SCF and ECS complexes (Pintard et al., 2004). Structurally, Cul3 has a highly elongated shape with two distinct functional domains. The N-terminus domain is composed of helical repeats and is responsible for the interaction with the BTB-containing protein, whereas the C-terminus harbors Rbx1 and Nedd8, and oversees the ubiquitin-conjugating enzyme recruitment (Wang et al., 2020).

Initially, it was thought that this E3 ligase was only involved in the anti-inflammatory pathway (Du et al., 1998), but after several years of research, it is
evident that its cellular role is broad. In fact, Cul3 has emerged as a critical player in the recognition and recruitment of numerous important substrates for ubiquitination. The Cul3-dependent ubiquitination process is associated with essential cellular processes, including oxidative stress, cell cycle progression, cell division, vesicle trafficking, and cell differentiation (Singer et al., 1999; Dubiel et al., 2017; Chen and Chen, 2016; Cheng et al., 2017). In humans, functional alterations of Cul3 have deleterious effects on the organism as a whole and usually lead to the development of several diseases, such as cancer, hypertension, and muscle disorders (Genschik et al., 2013).

*Rbx1*

RING box protein-1 (Rbx1), also known as a regulator of cullins-1 (Roc1), is an essential component of the Cullin E3 ubiquitin ligases. It consists of 108 amino acids with a C-terminus RING-H2 finger domain required for zinc ion binding and ligase activity (Chen et al., 2000). Rbx1 was initially identified as part of the Cul1-Roc1 ubiquitin ligase complex, but further analyses indicated that Rbx1 also binds to different cullin members, including Cul3 (Ohta et al., 1999). Functional characterization using various model systems demonstrated that Rbx1 is an essential gene for growth and development. In *Drosophila melanogaster*, Roc1a is required for cell proliferation and embryo development. In yeast, it is necessary for the ubiquitination of the cyclin-dependent kinase inhibitor Sic1 during the G1 to S cell cycle transition. While yeast cells are completely viable in the absence of Roc1
(Seol et al., 1999), *Drosophila melanogaster* Roc1a mutants die as late as first/second instars (Noureddine et al., 2002).

**BTB domain-containing proteins**

The BTB protein family was named after the discovery of a ~120 residue protein-protein interaction motif present at the N-terminus of the Broad-complex (BR-C), Tramtrack (Ttk), and Bric-à-brac (Bab) proteins of *Drosophila melanogaster* (Godt et al., 1993). Besides *Drosophila*, BTB domain-containing proteins have also been identified in several other species, such as *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens* (Geyer et al., 2003). The human genome encodes 188 BTB proteins divided into subfamilies according to their additional domains (Chaharbakhshi and Jemc, 2016; Stogios et al., 2005). Approximately 38 of 188 BTBs have been confirmed as Cul3 substrates adaptors (Zhuang et al., 2009).

The heterogeneity of substrates, substrates adaptors, intracellular and intercellular processes in which Cul3 is involved demonstrates this E3 ligase's relevance at both the cellular and organismal level. Although progress has been truly impressive over the past decades, there is still much that remains to be discovered to fully understand this intriguing class of Cullin-RING ubiquitin ligases, including the mechanisms of E2 recruitment and the potential role of the Cul3 substrate adaptors in that process, and the cellular effects of knocking Cul3 out in kidney cells. In the following sections, I discuss the mechanisms of E2 recruitment
as well as their interference in the ubiquitin-linkage type selection process. In addition, I also explain how Cul3 dysregulation leads to the development of a severe kidney disorder and discuss the implications of using a proteomics approach to identify novel Cul3 substrates in kidney cells.

**MECHANISMS OF E2 RECRUITMENT AND UBIQUITIN-LINKAGE TYPE SELECTION**

The process of conjugating a ubiquitin to a substrate is part of a highly regulated cellular system. It occurs through the formation of a covalent bond between α-carboxyl group of the terminus glycine (Gly) residue of ubiquitin and, typically, the ε-amino group of an internal lysine (Lys) residue of the substrate (Figure 1.6A). Cellular ubiquitin modifications occur in various forms, which are usually referred to as "Ubiquitin code" (Komander & Rape, 2012), and these modifications are interpreted by the cell according to the subcellular localization of the substrate, the number of ubiquitin peptides attached to the substrate, and the topology of the ubiquitin chain conjugated to the substrate (Pickart, 2001).

The attachment of a single ubiquitin moiety, known as monoubiquitination, is the most abundant ubiquitin modification. It regulates DNA repair, transcription, signal transduction, viral budding, endocytosis, and even proteasomal degradation (Chen & Mallampalli, 2009; Braten et al., 2016). Followed by the ubiquitin attachment to the ε-amino group of a target Lys, any of the eight amino groups of ubiquitin (Met1, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) can be utilized for the C-terminus attachment of another ubiquitin to form ubiquitin chains of
variable lengths, linkage types and configurations (homo- and heterotypic branched ubiquitin chains) (Xu et al., 2009; Zuin et al., 2014). Amongst the different homotypic ubiquitin chains, Lys48-linked ubiquitin chains were the first to be identified and are considered the most prevalent ones among all the homotypic poly-ubiquitin chains (Peng et al., 2003; Swatek & Komander, 2016). This type of linkage targets proteins for proteasome degradation, which regulates signal transduction, cell division, stress response, adaptive immune system, and development (Hershko and Ciechanover, 1998; Wang and Maldonado, 2006; Park et al., 2007).

The Lys63-linked ubiquitin chains are the second most abundant form of homotypic poly-ubiquitination (Davis & Gack, 2015). Different from Lys48-linked chains, this type of ubiquitin attachment does not target substrates to degradation. Instead, it regulates DNA repair or kinase activation, coordinates multiple steps of intracellular trafficking, and is involved in various forms of autophagy (Erpapazoglou et al., 2014). The remaining homotypic poly-ubiquitin chains are known as atypical, which include all variations of the poly-ubiquitin structure except for the classical Lys48 polyubiquitination. Although there has been a large body of data about Lys11 and Lys63, the other types of ubiquitin attachment represent a still-poorly understood set of molecular signals. More experimental data are needed to establish their cellular functions and mechanisms of chain assembly. (Figure 1.6B; Husnjak and Dikic, 2012).
Roles of E2s during ubiquitin chain assembly

The assembly of ubiquitin chains is usually initiated by transferring the first ubiquitin to a Lys residue on the substrate. Subsequently, the E2–E3 pair switches to chain elongation mode, in which additional ubiquitin proteins are attached to the substrate-linked ubiquitin. The attachment of another ubiquitin to the Lys residue in the substrate or the ubiquitin is often controlled by the E2s, which contradicts their early image as simple carriers of activated ubiquitin. Indeed, as discussed below, substantial evidence supporting their multiple cellular roles, which places them at a central position within the ubiquitination system.

First, E2s have the ability to determine the specificity of ubiquitin-linkage as well as the length of the attached ubiquitin chains. Some of them preferentially transfer ubiquitin to a Lys in the substrate to initiate ubiquitin chain formation, especially those of the UbE2D family (Kirkpatrick et al., 2006). In contrast, others are powerful chain-elongating factors (Windheim et al., 2008), which often depend on the type of linkage used to attach the first ubiquitin to the substrate. For instance, UbE2T attaches ubiquitin to multiple lysine residues in its substrate but lacks any ubiquitin chain extension activity (Alpi et al., 2008), whereas the Lys11-specific chain-elongating E2 UbE2S lack the capability for ubiquitin chain initiation (Williamson et al., 2009). Even though a few E2s can catalyze both tasks, such as the yeast E2 Cdc34 (Verma et al., 1997), it seems that a collaboration between chain-initiating and chain-elongating E2s is critical for the rapid assembling of poly-ubiquitin chains.
Secondly, E2s are also responsible for controlling the processivity of ubiquitin chain formation, which is defined as the number of ubiquitin molecules transferred to a growing chain during a single round of substrate association with an E3 (Ye & Rape, 2009). The faster the processivity of chain assembly, the higher the chances that the substrate will have a ubiquitin chain that is long enough to be recognized by the substrate receptors present in the proteasome or any other cellular structure. Thus, E2s have evolved several strategies to enhance the processivity efficiency, which includes recognizing specific substrate motifs for rapid ubiquitin chain initiation, oligomerization of charged E2s, and preassembly of ubiquitin chains on their active sites (Williamson et al., 2009; Brzovic et al., 2006; Li et al., 2007).

Lastly, several E2s can also connect ubiquitin molecules in a defined manner by modifying specific lysine residues in ubiquitin. This usually requires a non-covalent interaction between an E2 and the acceptor ubiquitin, which exposes a specific lysine on the acceptor ubiquitin to the active site of the E2 charged with the donor ubiquitin. For instance, UbE2K, UbE2R1, and UbE2G2 catalyze Lys48-linked chains, whereas UbE2N–UbE2V1 complex links ubiquitin molecules through Lys63 (Bremm & Komander, 2011; Choi et al., 2016; Stewart et al., 2016). Unlike the E2s mentioned above, UBE2D does not confer linkage specificity and instead synthesizes ubiquitin chains of all possible linkages (Brzovic et al., 2006). This preference for a specific Lys in ubiquitin is probably a result of the E2 orienting the acceptor ubiquitin in a way that exposes the chosen Lys to its active site (charged with the donor ubiquitin; Eddins et al., 2006).
In summary, by determining the length, the topology, and the processivity of ubiquitin chain assembly, E2s play a significant role in coordinating the outcomes of the ubiquitination process and, consequently, the fate of ubiquitinated proteins. However, despite all this progress, there are still many questions that remain answered, including:

(1) Can cullins (or E3 ligases) be involved in E2 selection?

(2) What other components besides Rbx1 can be used for the recruitment and binding to E2 enzymes?

(3) How does the BTB-Cul3-Rbx1 (BCR) complex select E2s?

Shedding more light on the mechanisms of E2 selection and recruitment as well as their interaction with the BCR complex will help improve our understanding of protein homeostasis and cellular signaling.

FAMILIAL HYPERKALEMIC HYPERTENSION (FHHt)

Familial hyperkalemic hypertension (FHHt) is an inherited disorder manifested by hyperkalemia and hypertension caused by the hyperactivation of sodium chloride cotransporters (NCC) that are expressed exclusively in the renal distal convoluted tubule (DCT; Mayan et al., 2002; Isobe et al., 2012). During the past two decades, considerable efforts have been taken to identify the proteins involved in FHHt, which revealed Cul3, its substrate adaptor KLHL3, and its substrates WNK1 and WNK4 as the major players in this disease. Autosomal
dominant mutations in Cul3 cause the most severe form of FHHt, but mutations in WNK1, WNK4, and KLHL3 can also lead to FHHt (Cornelius et al., 2018; Louis-Dit-Picard et al., 2012; Boyden et al., 2012).

The characterization of the functional roles of KLHL3, WNK1, and WNK4 in the regulation of NCC in the DCT has opened new and exciting areas of research that revolutionized our understanding of FHHt. However, a comprehensive understanding of all the roles of Cul3 in this disorder is far from reach. For instance, disruption of Cul3 in renal epithelia enhances WNK abundance and phosphorylation of NCC, but it also had other effects (Saritas et al., 2019). These included loss of expression of the water channel Aquaporin 2 leading to polyuria and renal injury (Suzuki et al, 2017). Moreover, Cul3 knockouts have unique phenotypes and phenotypic severities not seen in knockouts of its known substrates (Cornelius et al, 2018). In summary, other substrates and different pathways than Klhl3-Wnk1/Wnk4 are likely contributing to the phenotypic difference observed in Cul3 knockouts. In that regard, a comprehensive proteomics screen of Kidney-Specific (KS)-Cul3 knockout cells may help to identify candidate Cul3 targets and pathways that possibly contribute to the initial renal injury observed in FHHt. Furthermore, a proteomics approach is superior to screening by Western blot and immunofluorescence approaches since it circumvents testing and validating antibodies for the >40 known Cul3 adaptors.
DISSEMINATION SIGNIFICANCE

The heterogeneity of Cul3 substrates, intracellular and intercellular processes, and physiological systems demonstrates the importance of the Cul3-based E3 ligase. During the past two decades, considerable efforts have been taken to study Cul3, which has led to the discovery of the BCR complex (Pintard et al., 2004), the regulation by neddylation and deneddylation (Bosu and Kipreos, 2008), and the cellular processes in which the BTB domain-containing proteins are involved (Stogios et al., 2005). However, even with all this progress, much remains to be discovered to fully understand the nature of this intriguing regulatory axis.

The work presented here addresses some of the aspects of the function of Cul3 and has two primary goals: (1) elucidation of the mechanism of E2 recruitment and the potential role of the BTB domain-containing proteins in that process, and (2) identification of novel Cul3 substrates in kidney cells by taking advantage of newly acquired Cul3 knockout cells. This dissertation is organized into four sections: Chapter 1 provides background information on the ubiquitination system, Cul3-based E3 ligase, and its regulatory pathways. Chapter 2 describes a series of experiments focusing on a hypothesis that BTB domain-containing proteins help the complex identify substrates may also participate in the selection of the E2 enzyme. Chapter 3 describes a proteomics approach to testing the hypothesis that Cul3 regulates yet unidentified additional substrates in kidney cells. Finally, chapter 4 discusses future directions that should provide a further understanding of the mammalian Cul3-based E3 ligase.
**Figure 1.1 – The ubiquitin-dependent proteasome system:** This diagram illustrates the fundamental design of the ubiquitination process. The coordinated activity of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating enzyme (E3) is required for ubiquitin attachment to the target protein. Deubiquitinating enzymes (DUBs) remove ubiquitin from modified proteins or disassemble unanchored (free) ubiquitin chains. The polyubiquitinated protein is recognized and bound by the 19S subunit and degraded by the 20S subunit of the 26S proteasome.
Figure 1.2 – Structure of Ubiquitin refined at 1.8 Å resolution: This diagram depicts two different views of the peptide. **A) Structure view:** ubiquitin contains several secondary structures that are found throughout the peptide, including three and one half turns of α-helix, a short 310 helix, a mixed β-sheet with five strands and seven reverse turns. **B) Surface view:** ubiquitin contains a hydrophobic core, three hydrophobic residues found on the α-helix, and 11 of the 13 hydrophobic residues from the β-sheet are involved in constructing this hydrophobic core. The main contributor to the ubiquitin stability is the huge number of hydrogen-bonding interactions observed. The whole structure of ubiquitin undergoes significant hydrogen bonding, aside from the COOH terminus (Vijay-kumar et al., 1987).
Figure 1.3 – Overall structure of different E2s: Cartoon diagram of a variety of ubiquitin-activating enzymes. The four classes of E2s are colored as follows: class I in green, class II in light blue, class III in pink, and class IV in orange. The appendages are represented by the black lines.
Figure 1.4 – Structure of 3 classes of E3s and their mechanisms of ubiquitin transfer: E3 ligases are classified into three groups, depending on their mode of action. **RING-type E3 ligases (left)** transfer ubiquitin directly from an E2 cystine to a lysine residue of the target substrate. **HECT-type E3 ligases (middle)** conjugate ubiquitin in two steps, starting with a ubiquitin being transferred from an E2 cystine to a catalytic cystine of the HECT domain, followed by the transfer to a lysine residue of the target substrate. **RBR-type E3 ligases (right)** have a similar mechanism of HECT, despite being structurally different, and also conjugate the ubiquitin in two steps, starting with a ubiquitin being transferred from an E2 cystine to a catalytic cystine of the RING 2 domain, followed by the transfer to a lysine residue of the target substrate (Metzger et al., 2012).
Figure 1.5 – Structures of multisubunit Cullin RING-type ligase (CRLs) complexes: Diagrams of the Cul1 (A), Cul2 (B), Cul3 (C), Cul4 (D), Cul5 (E), and Cul7 (F). They are known as multisubunit complexes, which are composed of a scaffold protein known as Cullin, a substrate adaptor subunit on their N-terminus, and a Ring box protein on their C-terminus. They are generally inactive, but the covalent attachment of a protein called Nedd8 (N8) causes a conformational change that switches them on. Cul1 and Cul7, which are commonly known as SKP1-Cullin-F-Box (SCF) proteins, recruit substrates through a substrate adaptor subunit composed of Skp1 and an F-box protein. Cul2 and Cul5 recruit substrates through a substrate adaptor composed of Elongin-BC. Cul3 recruits substrates through a substrate adaptor subunit composed of BTB-domain-containing proteins. Cul4 recruit substrates through a substrate adaptor subunit composed of the DNA-damage-binding-protein 1(DDB1) and a DDB1-binding WD40 protein.
Figure 1.6 – Forms of ubiquitination: This diagram illustrates the different modes of ubiquitin conjugation. **A) General layout:** Ubiquitination can be either a single ubiquitin molecule attached to one or multiple residues of the substrate (monoubiquitination) or a chain of ubiquitin (polyubiquitination). Monoubiquitinated and multi-monoubiquitinated substrates are usually involved in signal transduction, while polyubiquitinated substrates have different fates depending on the lysine that ubiquitin is linked, and the length of the polyubiquitin chain formed. **B) Different forms of homotypic polyubiquitination:** Each ubiquitin chain contains a single linkage type that leads to distinct ubiquitin chain structures. Polyubiquitin chains linked by lysine 11 and 48 target proteins to the 26S proteasome for degradation and determine protein abundance in cells, while lysine 63-linked polyubiquitin chains are associated with signal transduction and endocytosis pathways.
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CHAPTER 2

Substrate selection subunits of the Cul3 E3 ligase participate in E2 binding and selection
INTRODUCTION

Ubiquitination is a process mediated by the sequential action of three different enzymes: a ubiquitin-activating (E1), a ubiquitin-conjugating (E2), and a ubiquitin-ligating (E3) enzyme. The coordinated activity of these three enzymes is required for the successful attachment of the ubiquitin to the target substrate (Figure 1.1; Hershko et al., 1983; Komander, 2009). Ubiquitin E3 ligases are considered the most critical components of this system because they are the ones that confer specificity to the ubiquitination process. Cullin-RING ubiquitin ligases (CRLs) represent the largest family of E3 ligase in eukaryotes, and among the seven CRLs coded in the human genome, Cullin3 (Cul3) is by far one of the best-understood (Deshaiies and Joazeiro, 2009; Morreale and Walden, 2016).

Cul3, a 768 amino acid-long polypeptide, is the core of a complex known as BTB-Cul3-Rbx1 (BCR), which also comprises the RING-box protein 1 (Rbx1) that is responsible for the ubiquitin-conjugating enzyme (E2) recruitment and a Bric-a-brac/Tramtrack/Broad protein (BTB) that functions as a bridge between Cul3 and the substrate (Ohta et al., 1999; Pintard et al., 2004; Stogios et al., 2005). Structurally, Cul3 has three domains, an N-terminus domain is composed of helical repeats that is responsible for the interaction with the BTB domain-containing protein, the C-terminus domain that binds Rbx1 and Nedd8, and is in charge of the ubiquitin-conjugating enzyme recruitment, and a stalk domain that connects them (Wang et al., 2020).
Cul3-dependent ubiquitination affects many substrates that are involved in a variety of cellular processes including oxidative stress, cell cycle progression, cell division, vesicle trafficking, and cell differentiation (Singer et al., 1999; Dubiel et al., 2017; Chen and Chen, 2016; Cheng et al., 2017). In humans, mutations in the Cul3 gene have deleterious effects and can lead to contributing to diseases such as cancer, hypertension, and muscle disorders (Genschik et al., 2013).

The heterogeneity of substrates, physiological systems, intracellular and intercellular processes in which Cul3 is involved demonstrates this E3 ligase’s relevance at both the cellular and organismal level. Although progress has been truly impressive over the past decades, there is still much that remains to be discovered to fully understand the structure and function of this intriguing class of Cullin-RING ubiquitin ligases. It has been determined by others that cullins bind E2s via the Rbx1 protein that is, in turn, bound to the cullin C-terminus. We reasoned that since substrates are always ubiquitinated with a specific type of branching (see chapter 1), the E2 selection must somehow “be aware” of the substrate identity. We suggest that the substrate selection subunit is also involved in E2 selection. Thus, we focused on testing the hypothesis that the N-terminus BTB domain-containing proteins bound to substrate collaborate with the C-terminus E2 enzymes to assist with substrate recognition.

In previous work in our lab, we developed cullin chimeras in which we fused the N- or C-terminus portion of Cul1 to the N- or C-terminus portion of Cul3 (Figure 2.1; Mitchell, 2014). In this work, we determined that the chimeric molecules had
the predicted binding characteristics based on their domain structure. We then determined that the wild type versions of Cul1 and Cul3 had differential preferences for different E2 enzymes by screening a large panel of human E2 proteins. Armed with that information, we observed that the cullin chimeras containing the N-terminus of Cul3 retained the binding ability of the wild-type Cul3 for BTB domain-containing proteins. Lastly, we tested the hypothesis that BTB domain-containing proteins either helped or were required for E2 binding and we determined that the substrate adapter BTB domain-containing protein was also required for the binding interaction to occur. Thus, our hypothesis that the E2 selection was, in part, mediated by the substrate adaptor was borne out.

RESULTS

Engineered cullin chimeras: The cullin-based E3 ligases’ basic architecture is shared amongst the seven known CRL complexes (Figure 1.5). Much attention has been placed on the substrate-recognition subunit (SRS) and the substrate selection process, but very little has been directed to the process of how E2s are selected. To better understand what makes each cullin complex unique, we used in this study two cullin chimeras with Cul1 and Cul3 that were previously designed and constructed by Jennifer Mitchell (2014). The two chimera constructs were named according to the domain of Cul3 that was replaced by that of Cul1. Thus, they are denoted Cul1 NTD and Cul1 CTD (Figure 2.1A). We tested the constructs for expression by transfection in mammalian cells, and they both expressed
normally and were neddylated as efficiently as their non-chimeric counterparts (Figure 2.1B). Neddylation is a good indication of proper folding of the C-terminus because it requires binding by Rbx1, binding by the Nedd8 E2 and proper orientation of the domain to occur (Read et al., 2000; Pintard et al., 2003; Pan et al., 2004).

**The chimera possessing the N-terminus of Cul3 binds BTB adapter proteins:** The observation that the chimeric proteins were neddylated provides evidence that they are probably folding properly at their C-terminus domains. We then tested the chimeras’ N-terminus domain binding specificity by examining binding to substrate adaptor subunits, BTB domain-containing proteins for Cul3 and Skp1 for Cul1. As we hypothesized, the chimeras containing the Cul3 N-terminus domain bound the BTB domain-containing proteins Kctd6 and Klhl3, as well as the Cul3 wild-type construct, (Figures 2.2A and 2.2B, lanes 3 and 5), whereas the constructs containing the Cul1 N-terminus did not (Figures 2.2A and 2.2B, lane 4). Conversely, we tested for binding to Skp1, and only the Cul1 wild-type and Cul1 N-terminus constructs bound it (Figure 2.2C, lanes 2 and 4). These data are consistent with the N-terminus folding properly and demonstrated that the chimeras maintained the specificity of donor cullin domains.

**Cul1 and Cul3 exhibit differential binding to E2 enzymes:** E2 enzymes have been shown to interact with the highly conserved C-terminus domain of cullins via the RING protein 1 (Rbx1). They work together with CRLs to attach ubiquitin molecules to substrates and regulate both the topology of the polyubiquitin chains
(that is, they determine the type of branching and therefore the fate of the substrate) and the polyubiquitination reactions' processivity. There are 40 ubiquitin-conjugating enzymes encoded in the human genome (Figure 1.3). While a growing number of studies have characterized different E2-E3 pairs, little is known about the specificity of these interactions. Here we investigated the binding interactions between E2 enzymes and wild type Cul1 or Cul3 in transfected cells to begin to create a picture of the potential binding interactions of the wild type enzymes. We observed that two E2 enzymes (UbE2B, and UbE2F) bound to both Cul1 and Cul3 (Figure 2.3B), while five others (UbE2C, UbE2D1, UbE2D2, UbE2E2, and UbE2G1) did not bind to either cullin (Figure 2.3A). However, two of the ones we examined (UbeE2E1 and UbE2E3) displayed selectivity: UbE2E3 bound Cul1 and not Cul3 and UbE2E1 bound Cul3 and not Cul1 (Table 2.1). This selectivity was then explored further by mapping the regions of Cul3 required to specifically select an E2.

The BTB binding region of Cul3 is required for binding to E2 enzymes: Since we had observed that UbE2E1 binds to exclusively to Cul3, we wanted to determine which part of Cul3 was required for UbE2E1 binding. To investigate this, we performed co-transfections and co-immunoprecipitation experiments to examine binding interactions between this E2 and chimeric cullins or a Cul3 mutant that cannot bind to BTB domain-containing proteins. The current model of CRL assembly suggests that E2 enzymes interact with cullin-based E3 ligases via interaction with the ring domain protein Rbx1 in the C-terminus domain of cullins.
Since UbE2E1 was able to bind to Cul3 and not to Cul1, we expected that the Cul3 chimera containing the C-terminus of Cul1 would not be able to bind this E2. We were surprised to see the opposite (Figure 2.4, lane 1). This observation suggests that the N-terminus domain of Cul3 confers specificity for binding to this E2.

Further, we expected that replacing the N-terminus domain of Cul3 with that of Cul1 would have little effect on the binding of this E2, as the current model suggests that the N-terminus domain is not involved. Surprisingly, replacing the N-terminus domain of Cul3 with Cul1 diminished the binding (Figure 2.4, compare lanes 1 and 2). Moreover, the Cul3 mutant lacking residues involved in BTB protein binding (Cul3Δ51-67) also significantly diminished binding (Figure 2.4, compare lanes 1, 2, and 3). Taken together, these results suggest that the N-terminus, specifically the BTB binding region, is required for the binding of the UbE2E1 to Cul3. This is consistent with our hypothesis that the BTB substrate adapters or the N-terminus domain of Cul3 itself are somehow involved in recruiting certain E2s to the Cul3 complex.

**UbE2E1 is not likely a Cul3 substrate:** Alternatively, another possibility is that UbE2E1 is not serving as a ubiquitin-conjugating enzyme with Cul3 but is instead bound to Cul3 as a substrate (See model, Figure 2.5A and 2.5B). Since substrates are recruited to the Cul3 complex by BTB proteins in the N-terminus domain, this could explain the observation that the BTB binding domain of Cul3 is required for this E2 to bind. We have examined this in many ways for Cul3 substrates in the past, including co-transfection to determine if levels of the substrate decrease
when Cul3 is present and if it is ubiquitinated, as well as transfection into WT vs Cul3 KO cells (McEvoy et al., 2007; Davidge et al., 2017). Here we show the results of one such experiment in which we examined UbE2E1 expression levels in wild-type and Cul3 knockout (KO) cells. As shown in Figure 2.5, the amounts of UbE2E1 are the same in the presence and absence of Cul3, which would be consistent with the idea that Cul3 is not likely to be involved in UbE2E1 degradation (Figure 2.5C, compare lanes 1 and 2).

**BTB adapter proteins can bind E2s:** Since our hypothesis stated that the BTB domain-containing protein that acts as a substrate adaptor may also participate in E2 binding to help select the correct E2, we examined the ability of UbE2E1 to bind four BTB domain-containing proteins, Kctd6, BTBD1, SPOP, and KLHL3, that were previously shown to interact with Cul3 (Xu et al., 2002; Ohta et al., 2013; Smaldone et al., 2015; Gladwyn-Ng et al., 2015; Gschweitl et al., 2016). We found that UbE2E1 bound to only Kctd6 (Figure 2.6, lane 2). This experiment revealed a novel interaction between E2 enzymes and BTB domain-containing proteins that have not been observed before. Therefore, this result is consistent with our hypothesis that there is selective binding between BTB proteins and E2s.

**Cul3 requires the BTB adapter to bind E2s:** In order to examine the functional role of this novel interaction we chose to look at the cyclin E ubiquitination process that we discovered and have extensively characterized (Singer et al., 1999; Davidge et al., 2017). RhoBTB3 is a BTB domain-containing protein that is a component of Cul3-dependent E3 ubiquitin ligase complex and has been shown
to target cyclin E for degradation (Lu and Pfeffer, 2013). However, our group showed that cyclin E could also bind to Cul3 directly and independently of RhoBTB3 (Davidge et al., 2019). Since RhoBTB3 is not essential for the Cul3/cyclin E interaction, we speculated that it might be playing another role in the active complex. We hypothesized that since specific E2 recruitment should play an essential role for the E3 ligase, RhoBTB3 could be responsible for recruiting the E2 enzyme that ubiquitinates cyclin E under certain circumstances. If BTB proteins are involved in E2 selection, it will ensure that the proper E2 is recruited to form the correct ubiquitin linkage for a given substrate. To test this hypothesis, we examined the binding of Cul3 and RhoBTB3 to the E2 enzyme UbE2E1.

Consistent with our hypothesis, we observed that both RhoBTB3 and Cul3 could bind to UbE2E1 (Figures 2.7A lane 2 and 2.7B lane 2, respectively). We then examined if Cul3 binding to UbE2E1 requires the BTB domain interacting region on Cul3. We observed that UbE2E1 could not bind the Cul3 mutant that is incapable of binding to BTB proteins, Cul3Δ51-67 (Figure 2.7B, lane 3). This indicates that the interaction with the E2 enzyme is likely to be entirely mediated by RhoBTB3. To confirm that Cul3 does not mediate the interaction between RhoBTB3 and UbE2E1, Cul3 knockout (KO) cells (Ibeawuchi et al., 2015) were used to analyze binding of RhoBTB3 and UbE2E1 to determine if Cul3 was essential for this interaction. As can be seen in lanes two and four of figure 2.7A, RhoBTB3 binds to the E2 enzyme in the absence of Cul3 (Figure 2.7A, lane 4) to the same degree that the two proteins bind in the presence of Cul3 (Figure 2.7A,
lane 2), indicating that Cul3 is not required for the BTB to interact with the E2. Together these results demonstrate that a BTB protein, in this instance RhoBTB3, can interact with an E2 enzyme independently of Cul3 and may enhance E2 binding in vivo. Next, we tested the hypothesis that RhoBTB3 in combination with UbE2E1 boosts the degradation of cyclin E in vivo.

**Substrate levels reduced in the presence of Cul3-BTB-E2 complex:** To test this hypothesis, we performed co-transfections experiments to examine the repercussions of RhoBTB3 and UbE2E1 overexpression on cyclin E degradation. As shown in Figure 2.8, the expression levels of cyclin E are reduced in all lanes in which UbE2E1 is present, indicating that this E2 might boost the degradation of cyclin E (Figure 2.8, lanes 3, 5, 7, 8, and 9). We also observed that in the presence of the Cul3-RhoBTB3-UbE2E1 complex, cyclin E levels are 50% lower than the lane that contains the Cul3 Δ51-67 mutant that is incapable of binding to BTB proteins (Figure 2.8, compare lanes 8 and 9). These results suggest that the recruitment of the correct E2 by the BTB adapter may enhance the degradation of the substrates.

**DISCUSSION**

The ubiquitin-dependent proteasome system (UPS) is an elaborate and highly regulated cellular system that controls the attachment of ubiquitin to a target substrate. Once ubiquitin is attached to the substrate, seven internal lysine residues (Lys6, Lys11, Lys27, Lys33, Lys29, Lys48, and Lys63) (Xu et al., 2009)
on the ubiquitin molecule provide conjugation sites for other ubiquitin molecules and lead to the formation of different polyubiquitin chains that range from a more linear (Lys6, Lys11, and Lys63) to a more compact and branched (Lys27, Lys29, Lys33, and Lys48) conformation (Pickart, 2001). This diversity in architecture of shape is known as the “ubiquitin code” and, through the recognition of specific ubiquitin receptors, determines the fate of the ubiquitinated substrates (Husnjak and Dikic, 2012). For example, a polyubiquitin chain with lysine 48 linkages is branched and a signal for protein degradation (Chau et al., 1989; Thrower et al., 2000), while lysine 63 linkages are linear and involved in the DNA repair pathway (Figure 1.2; Spence et al., 1995; Hofmann and Pickart, 1999; Hoege et al., 2002). Ubiquitin-conjugating enzymes (E2s) play a critical role in this lysine selection process, as they regulate the position of the lysine residue chosen for the attachment and the type of ubiquitin linkage that will be produced (Valimberti et al., 2015).

UbE2E1, also known as UbcH6, attaches ubiquitin to substrates using lysine 48. Studies have been shown this E2 can interact with multiple proteins, such as Ataxin 1, NEDD4, and TRIM21 (Hong et al., 2008; Anan et al., 2001; Anandapadamanaban et al., 2019), is involved in different physiological processes (Plafker et al., 2004; Malakhova & Zhang, 2008; Hong et al., 2008) and has been recently identified as a prognostic factor in acute myeloid leukemia (Luo et al., 2016). Despite the considerable efforts that have been taken to study this E2, little is known about the specificity of interactions between this E2 and different cullins.
Here, we have demonstrated that UbE2E1 preferentially binds Cul3 and is unable to interact with Cul1 (Figure 2.3), and the region of Cul3 required for binding to UbE2E1 is the BTB domain binding region, not the C-terminus domain, as has been suggested by the current models (Petroski & Deshaies, 2005; Bosu & Kipreos, 2008; Deshaies & Joazeiro, 2009; Metzger et al., 2014). Thus, these observations do not align with the current model of how cullin E3 ligases interact with E2s. Some precedence for alternative E2 recruitment has been shown by our group, however this was only demonstrated in bacterially expressed proteins (Plafker et al., 2009). The same study also demonstrated that UbE2E1 and UbE2E2 bind the N-terminus of Cul3 in vitro, suggesting that interaction with the N-terminus of cullin complexes may be a trait shared by all class III E2s.

Our study also revealed a previously unreported binding interaction between UbE2E1 and the BTB domain-containing protein Kctd6 (Figure 2.6). The observations that UbE2E1 requires the BTB binding region to bind Cul3 and interacts with the Cul3 substrate adapter Kctd6 can be explained in more than one way. One possibility is that UbE2E1 is not serving as a ubiquitin-conjugating enzyme but is instead recruited by Cul3/Kctd6 as a substrate. We partially addressed this possibility by showing that the expression levels of UbE2E1 in wild type and Cul3 KO cells are the same, thus Cul3 probably does not participate in the UbE2E1 ubiquitination and degradation (Figure 2.7). Another possibility, supporting our model, is that UbE2E1 is recruited to the Cul3 complex by specific BTB domain-containing proteins such as Kctd6. Thus, we suggest that certain BTB
domain-containing proteins may bind both the E2 and the substrate, ensuring proper ubiquitin branching.

Here, we demonstrated that RhoBTB3 is also able to bind to UbE2E1, both in the presence and absence of Cul3, and this interaction diminishes the cellular levels of cyclin E (Figure 2.8). These findings are significant as they confirm that Cul3 substrate adaptors participate in the E2 selection process to mediate a functional complex. This also would help to explain how Cul3 can form a variety of ubiquitin chain linkages as different E2s are associated with this process. The revelation that the Cul3\Delta51-67 mutant, which cannot bind BTB proteins, is incapable of binding UbE2E1 (Figure 2.7) presents a second piece of information supporting the idea that BTB domain-containing proteins are necessary for E2 selection to occur.

Until now, models have suggested that the recruitment of E2s for the ubiquitin-conjugation process occurs independently of the substrate that is being ubiquitinated and involves the C-terminus of Cul3 interacting with the ubiquitin-conjugating enzyme (E2) and the N-terminus interacting with and recruiting the substrate via a BTB domain-containing protein (Figure 2.9A). In the work reported here, we have made four important discoveries about this subject. First, we demonstrated that Cul1 and Cul3 have differential binding preferences for E2s (Figure 2.7). Second, we determined that the cullin chimeras containing the N-terminus of Cul3 retained the binding ability of the wild type Cul3 (Figures 2.2 and 2.4). Third, UbE2E1 preferentially binds to Cul3 rather than Cul1 and a BTB
adaptor protein is required for this interaction to occur (Figures 2.4, 2.7, and 2.8). Fourth, certain E2 enzymes might be able to interact with both termini of Cul3 (Figure 2.9B).

Lastly, several experiments remain to be done to further characterize and understand this novel selection mechanism. For example, a crucial question that needs to be addressed is what, if any, role does the previously characterized Rbx1 recruitment play in E2 selection? We have the tools to examine this question, a mutant that does not bind Rbx1 was created and characterized in our laboratory and would be an invaluable tool for such an analysis (Wimuttisuk & Singer, 2007). Another important question that needs to be answered is what region of the N-terminus Cul3 is responsible for the interaction with the E2s? Besides the mutant that cannot bind to Rbx1, we have at our disposal a variety of other mutants with specific deletions that would be very helpful in pinpointing the binding region.

MATERIAL AND METHODS

Cell culture and transfections: Cells (HEK293 wild type and Cul3 knockout) were maintained in DMEM (Gibco™, CAT no. 11965092) supplemented with 10% Fetal Bovine Serum (Atlas Biological, CAT no. F-0500-A), 1% L-Glutamine (Gibco™, CAT no. 25030081), and 1% Penicillin/Streptomycin (Gibco™, CAT no. 15070063). Cells were split 1:20 for transfection in 6 cm dishes the night before transfection. Transfections were performed using the calcium phosphate precipitation (Jordan et al., 1996). For immunoprecipitations and Western blots,
cells were harvested 48 hours post-transfection. The amount of plasmid DNA transfected into each plate varied from 1 to 10μg. All transfected cells were scrapped and harvested using 600μL of a solution containing 10mL of radioimmunoprecipitation assay lysis buffer (RIPA) mixed with 1 protease inhibitor tablet (Thermo Scientific™, CAT no. PIA32953), and then sonicated with an ultrasonic cell disrupter (VIRTIS VIRSONIC 100) for 15 seconds at 50% power before being used for immunoprecipitations or western blots. CRISPR knockout Cul3 HEK293 cells were a gift from Curt Sigmund.

Western blotting and immunoprecipitations: Western blots and immunoprecipitations were conducted as previously described (Wimuttisuk et al., 2014). In short, 500μL of the sonicated transfection lysate was added to the desired antibody in a microcentrifuge tube. 40μl of RIPA–Sepharose beads (Thermo Scientific™, CAT no. 101041) were then added to the mixture, and the immunoprecipitations were placed on a rotator for 2 h at room temperature before being rinsed with RIPA buffer for 15 seconds at 1500rpm, heated in 15μL of SDS-PAGE loading buffer for 2 minutes, and ran on an SDS-PAGE gel. The following antibodies were used for immunoprecipitations at 1–2μg or at the indicated dilution for western blotting: monoclonal anti-FLAG (1:1000, CAT no. F3165, Sigma-Aldrich), monoclonal anti-Myc (9E10) (1:500, c-Myc mouse anti-Human, CAT no. MA1-980, Invitrogen™), polyclonal anti-c-Myc (A14) (1:500, Santa Cruz Biotechnology), S-peptide monoclonal antibody (1:1000, clone 6.2, CAT no. MA1-981, Invitrogen™), monoclonal anti-HA.11 (16B12) (1:1000, CAT no. MMS-101P-
200, BioLegend), polyclonal anti-HA (1:1000, CAT no. PA1-985, Invitrogen™), polyclonal anti-β actin (CAT no. AM4302, Invitrogen™), polyclonal anti-Cul3 (Singer et al., 1999; McEvoy et al., 2007), and monoclonal anti-cyclin E (HE12) (1:100 for transfected samples, CAT no. 32-160-0, Invitrogen™), Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (1:10000 for transfected samples, CAT no. 31460, Invitrogen™), Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (1:10000 for transfected samples, CAT no. G-21040, Invitrogen™). Western blots were visualized using myECL™ Imager (CAT no. 62236, Thermo Scientific™).

**Plasmids:** 3x-Flag-Cul3 (Sweeney et al., 2020) was used for all Cul3-containing transfections, including the Cul3 mutant (Cul3Δ51-67). BTB domain-containing proteins were expressed either using the CS2-Myc-tagged or the CS2+S-tagged expression vectors (Znosko et al., 2010). E2 enzymes were expressed either in CS2+HA tagged or CS2+Myc-tagged expression vectors. The chimeric cullins were cloned into the pCMV-3Tag-6 vector (Mitchell, 2014).

**Engineered cullin chimeras:** The two cullin chimeras used in this study were previously designed and constructed by Jennifer Mitchell, 2014.

**Quantification of UbE2E1 and cyclin E relative levels:** The ImageJ software was used for the quantification of the relative levels of UbE2E1 and cyclin E bands shown in figures 2.5 and 2.8, respectively. The quantification reflects the relative amounts as a ratio of each protein band relative to the lane’s loading control. In
the UbE2E1 blot, the loading control is in lane 1 (Figure 2.5) and in the cyclin E blot, the loading control is in lane 1 (Figure 2.8). ImageJ is a Java based (runs on all operating systems) freeware by Wayne Rasband from National Institute of Health (USA) and is available for download at: http://rsb.info.nih.gov/ij/
Figure 2.1 – Engineered cullin chimeras: (A) Schematic of wild-type and engineered Cullin chimeras (Mitchell, 2014). The two chimera constructs were named according to the domain of Cul3 that was replaced by that of Cul1. Thus, they are denoted Cul1 NTD (N-terminus domain chimeric cullin) and Cul1 CTD (C-terminus domain chimeric cullin). (B) Western blot of cell extracts from HEK293 cells transfected with Cul1, Cul3, Cul1 NTD, Cul1 CTD with an anti-FLAG antibody.
Figure 2.2 – Chimeras binding to substrate adapters proteins: Western blots were performed on immunoprecipitants and whole cell extracts from HEK293 cells transformed with FLAG-tagged Cul1, Cul3, Cul1 NTD and Cul1 CTD. Panel A: Cells also transfected with Myc-tagged Kctd6. Panel B: Cells also transfected with Myc-KLHL3. Panel C: Cells also transfected with HA-Skp1. Top rows show immunoprecipitation with an anti-FLAG antibody and Western blot with an anti-Myc antibody (Panel A and B), or ant HA-antibody (Panel C). Second rows show control precipitation with an anti-FLAG antibody. Third rows show control Western blot with anti-Myc antibody (Panel A and B), and anti-HA antibody (Panel C). Fourth Row, control Western Blot with anti-FLAG antibody.
### A

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Page 82
Figure 2.3 – Cul1 and Cul3 exhibit differential binding to E2 enzymes: HEK 293 cells were co-transfected with Myc-tagged E2 enzymes and FLAG-tagged Cul1 or Cul3. Cells were harvested after 48 hours. Immunoprecipitation was carried out using a FLAG antibody, and immunoblotting was used to probe for Myc-E2 binding. (A) E2s that could not bind to either Cul1 or Cul3 (top row). (B) E2s that could bind to both Cul1 and Cul3 (top row). (C) E2s that could bind only Cul3 (top row) or only Cul1. The second rows on A show control precipitation with an anti-FLAG antibody. The lower 2 rows show the expression of the Myc-E2 and FLAG-cullin, respectively.
Table 2.1 – List of ubiquitin-conjugating (E2) enzymes E2 clones into mammalian expression vector that were used in the differential binding experiments: Column one shows the names of the ubiquitin-conjugating enzymes, column two shows the classes to which they belong, column three shows the lysine they use to attach the ubiquitin to the substrate (Komander & Rape, 2012), and column four shows the differential binding to Cul1 and Cul3 based on co-immunoprecipitation (Figure 2.3).

<table>
<thead>
<tr>
<th>E2 enzyme</th>
<th>Class</th>
<th>Ubiquitin attachment</th>
<th>Binding to Cul1</th>
<th>Binding to Cul3</th>
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<tbody>
<tr>
<td>UbE2B</td>
<td>1</td>
<td>K11, K48, K63</td>
<td>+</td>
<td>+</td>
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<tr>
<td>UbE2D1</td>
<td>1</td>
<td>K48</td>
<td>-</td>
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<td>K48</td>
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<td>K48</td>
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<td>UbE2F</td>
<td>2</td>
<td>Nedd8</td>
<td>+</td>
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<td>UbE2G1</td>
<td>1</td>
<td>K48, k63</td>
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Figure 2.4 – Cul3 requires the BTB binding region to interact with E2: Western blot of FLAG-tagged chimeric cullins, and a Cul3 mutant transfected in HEK 293 cells with UbE2E1. Cells were harvested after 48 hours. Immunoprecipitation was carried out using a FLAG antibody, and immunoblotting was used to probe for Myc-UbE2E1 binding (top panel). The second panel is a FLAG antibody control and verifies that the immunoprecipitation pulled down FLAG-cullins. The bottom two panels indicate expression levels of Myc-UbE2E1 and FLAG-cullins, respectively. Reprinted from "Characterization of Functional Domains of Cul3, an E3 Ubiquitin Ligase, Using Chimeric Analysis", by Jennifer Anne Mitchell, 2014, M.Sc. thesis, Biology Department, Portland State University, p. 67.
Figure 2.5 – E2 enzyme UbE2E1 is likely not a Cul3 substrate: (A) Current model for the cellular role of UbE2E1, showing UbE2E1 acting as a ubiquitin-conjugating enzyme. (B) Alternative model for the role of UbE2E1, showing UbE2E1 acting as a Cul3 substrate. (C) HEK 293 WT and Cul3 KO cells (See Chapter 3) were transfected with Myc-tagged UbE2E1. Cells were harvested after 48 hours, and Western blot was carried out using Myc antibody (bottom panel). The first row indicates expression levels of endogenous Cul3. Second row shows the levels of UbE2E1 in the presence and absence of Cul3 (lanes 1 and 2). Third row indicates the quantification of UbE2E1 levels in WT and Cul3 KO cells.
Figure 2.6 – BTB adapter proteins can bind E2s: HEK 293 cells were co-transfected with HA-tagged Ube2E1 and four different Myc-tagged BTB domain proteins (Kctd6, BTBD1, SPOP, and KLHL3). Cells were harvested after 48 hours. Immunoprecipitation was carried out using Myc antibody, and immunoblotting was used to probe for HA-Ube2E1 binding (top row). The bottom two rows indicate expression levels of Myc-BTB proteins and HA-Ube2E1, respectively. Ube2E1 only binds to Kctd6 (lane 3). Reprinted from “Characterization of Functional Domains of Cul3, an E3 Ubiquitin Ligase, Using Chimeric Analysis”, by Jennifer Anne Mitchell, 2014, M.Sc. thesis, Biology Department, Portland State University, p. 68.
Figure 2.7 – Cul3 requires a BTB protein to interact with E2: (A) RhoBTB3 was co-transfected with the E2 ubiquitin-conjugating enzyme HA-tagged UbE2E1 in the presence of endogenous Cul3 (WT cells, lanes 1 and 2) and absence of Cul3 (KO cells, lanes 3 and 4), and checked for binding. RhoBTB3 binds UbE2E1 in both cell types. (B) The UbE2E1 was co-transfected with WT FLAG-Cul3 and FLAG-Cul3Δ51-67 and checked for binding. WT Cul3 (lane 2) but not Cul3Δ51-67, which cannot bind BTB proteins (lane 3), can bind the E2 enzyme UbE2E1. Reprinted from “The Cul3 Ubiquitin Ligase: An Essential Regulator of Diverse Cellular Processes”, by Brittney Marie Davidge, 2017, Ph.D. dissertation, Biology Department, Portland State University, p. 54.
Figure 2.8 – UbE2E1 collaborates with the ubiquitination and subsequent degradation of cyclin E: HEK 293 cells were co-transfected with Myc-tagged Cyclin E, HA-tagged UbE2E1, FLAG-tagged Cul3 or Cul3 Δ51-67, and S-tagged RhoBTB3. Immunoblotting was used to probe for HA-UbE2E1 (top row), FLAG-Cul3 or Cul3 Δ51-67 (second row), and S-tag-RhoBTB3 (third row), and Myc-Cyclin E (fourth row). Quantification of the western blot bands (bottom row).
Figure 2.9 – A new model for Cul3 complex assembly: (A) Current suggested model of Cul3 complex assembly. Rbx1 recruits the E2s to the BCR complex and the substrate is ubiquitinated. This recruitment is non E2 specific. (B) Proposed model of an E2 assembly with Cul3 with the help of a BTB domain-containing protein. A BTB adapter protein is required for the interaction between Cul3 and E2s, which implies that the BTB domain-containing proteins are likely to be involved in the E2 recruitment and certain E2 enzymes can interact with both termini of Cul3.
ACKNOWLEDGMENT

The author would like to thank Jennifer Anne Mitchell and Brittney Marie Davidge, for their valuable contributions to this study.
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CHAPTER 3

Identification of novel kidney specific Cul3 degradation substrates
INTRODUCTION

Ubiquitination is a process mediated by the sequential action of three different enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. The coordinated activity of these three enzymes is required for the successful attachment of the ubiquitin to the target substrate (Hershko et al., 1983; Komander, 2009). Cullin-RING ubiquitin ligases (CRLs) represent the largest family of E3 ligases in eukaryotes. Among the seven CRLs coded in the human genome, Cullin3-based E3 ligase (Cul3) is one of the most-studied and well-understood enzymes (Deshai and Joazeiro, 2009; Morreale and Walden, 2016;).

Cul3 is a highly conserved gene that is expressed in all human organs (The Human Protein Atlas, 2021; Uhlen et al., 2015; Thul et al., 2017). It is the core piece of the BTB-Cul3-Rbx1 (BCR) complex, which also comprises the RING-box protein 1 (RBX1) that is responsible for the ubiquitin-conjugating enzyme (E2) recruitment and a Bric-a-brac/Tramtrack/Broad complex (BTB) protein that functions as a bridge between Cul3 and the substrate (Ohta et al., 1999; Pintard et al., 2004; Stogios et al., 2005).

Several studies have established that Cul3 regulates the progression of many critical cellular processes by targeting proteins either for 26S proteasome-mediated degradation or for non-proteolytic cellular pathways, such as chromosome segregation, development, cytoskeletal remodeling, and response to
DNA repair (Dubiel et al., 2017; Chen and Chen, 2016; Cheng et al., 2017; Furukawa et al., 2003; Chen et al., 2009; Sumara et al., 2007; Moghe et al., 2012; Mulvaney et al., 2016). Its ultimate disruption results in early embryonic lethality (Singer et al., 1999). Mutations have been associated with the development of several diseases, such as epithelial cancers, renal dysfunctions, neurodegenerative and muscle disorders (Popovic et al., 2014). Disruption of Cul3 was also associated with increased lysine deficient protein kinase (WNK) abundance and phosphorylation of the thiazide-sensitive NaCl cotransporters (NCC) in renal epithelia (Cornelius et al., 2019). These included loss of expression of the water channel Aquaporin 2, a vasopressin-regulated water-channel protein, leading to polyuria, renal injury, and dehydration (Suzuki et al, 2017). Importantly, Cul3 has also been associated with Familial Hyperkalemic Hypertension (FHHt), also known as pseudohypoaldosteronism II (Shiba et al., 2013; Genschik et al., 2013; Cuneo and Mittag, 2019).

FHHt is an inherited disorder characterized by hyperkalemia and hypertension caused by the hyperactivation of sodium chloride cotransporters (NCC) that are expressed exclusively in distal convoluted tubules of the kidney (DCT; Mayan et al., 2002; Isobe et al., 2012). Autosomal dominant mutations in Cul3 cause the most severe form of FHHt, but mutations in WNK1, WNK4, and KLHL3 can also lead to FHHt (Cornelius et al., 2018; Louis-Dit-Picard et al., 2012; Boyden et al., 2012). During the past two decades, considerable efforts have been taken to identify the proteins involved in FHHt. A solid body of work revealed Cul3,
its substrate adaptor KLHL3, and its substrates WNK1 and WNK4 as the major players in the disease (McCormick et al., 2014).

Cul3 knockouts shown a unique phenotype severity in the kidney not seen in knockouts of its known substrates (WNK1, WNK4; McCormick et al., 2014; Cornelius et al., 2018). Taken together, we hypothesized that there are other contributing factors besides Klhl3-Wnk1/Wnk4 pathway that leads to this phenotypic difference. Hence, I proposed to perform an unbiased proteomic screen to identify candidate Cul3 targets and pathways that may contribute to the initial renal injury observed in Kidney Specific (KS)-CUL3(-/-) mice, in which Cul3 was deleted only along the nephron. Our group has also observed an increased abundance of two adaptors with known renal functions, KLHL3 and Keap1, in Cul3 KO cells (McCormick et al., 2014; Watai et al., 2007), thus my approach may also help to identify more adaptors expressed in the kidney. Therefore, I predict that CRL disruption would increase the abundances of other renal CRL adaptors in addition to other potentially important substrates. A proteomics approach is superior to screening by Western blot and immunofluorescence approaches since it circumvents testing and validating antibodies for the >40 known CRL adaptors. I also hypothesized I would identify novel substrates such as aquaporins or regulators of their activity.
RESULTS

Validation of the HEK293 wild type and Cul3 knockout cells: HEK293 which was first derived from embryonic kidneys became a widespread non-cancer cell model that allowed researchers to use it for KO studies. (Russell et al., 1977). For my studies, I used wild-type (WT) and a HEK293 variant (Cul3 KO) with a Cul3 deletion performed by CRISPR technology (Ibeawuchi et al., 2015). We have previously shown that Cul3 KO cells have elevated levels of several known Cul3 substrates (McEvoy et al., 2007; Davidge et al., 2019). In addition to the classic molecular techniques previously used, I decided to validate the actual cells we used for the Mass Spectrometry (MS)-based proteomics screen by examining the endogenous levels of Cul3 in WT and Cul3 KO HEK 293 cells (Figure 3.1A, top panel). By using Western blots (WB), I observed that the Cul3 levels were dramatically reduced in Cul3 KO cells. The residual bands that I observed on WBs were probably a slight amount of cross-reactivity to all the other cullins that run in the same location on the gel.

To ascertain the change of Cul3 function in the WT vs Cul3 KO cell lines, both cell lines were transfected with MYC-cyclin E, a well-characterized substrate of Cul3, to determine its half-life (Figure 3.1B, top panel; Davidge et al., 2019). Following cycloheximide (CHX) addition, which inhibits protein synthesis in eukaryotic cells, I harvested the cells every 3 hours for 24 hours. I observed that cyclin E has a half-life of about 3 hours in the WT cells but a half-life of about 6
hours in the KO cells (Figure 3.1C). This change is similar to the change we have observed in mouse fibroblasts that have a Cul3 deletion (McEvoy et al., 2007), leading to an increase in the half-life of cyclin E.

**Quantitative proteomics analysis of cells lacking Cul3:** A MS-Based Label-Free Quantitative Proteomics analysis (Zhu et al., 2010) was performed on four biological replicates of each cell line, as summarized in Figure 3.2. I compared the relative amount of proteins of the WT and Cul3 KO cells, focusing my attention on proteins that had spectral counts in all replicates. As shown in Tables 3.1 and 3.2, approximately 2900 proteins and 13000 unique peptides, respectively, were identified in each of the eight biological replicates (four WT and four KO) used in this study. Low data variability and no statistically significant p-values confirm the reproducibility of the analysis.

**Main results of the proteomics analysis:** Figure 3.3 summarizes the data obtained from the MS analysis of both the WT and Cul3 KO cells. The total number of proteins identified and quantified was 4087. Of those, 3265 were identified in both cell lines, and only these were considered for subsequent statistical analyses. We ordered them based on their calculated fold changes (comparing the two genotypes), performed a t-test analysis, and used p < 0.05 as the cutoff for the p-value. These three criteria combined were used as the threshold for up- or downregulation of the proteins identified in our procedure. For instance, if the p-value was lower than 0.05 and the fold change was lower or larger than 1.5, we
considered that the protein was differentially expressed. The number of proteins whose statistically significant fold change was at least 50% compared to the observed in the WT (p-value ≤ 0.05) was 259. Of these, 77 of the proteins were upregulated with a fold change ≥ 1.5, and 46 were downregulated with a fold change ≤ -1.5.

**Functional categorization of proteins whose spectral counts were significantly high or low in KO cells:** I used Gene Ontology (GO), a tool that provides a framework and set of concepts for describing the functions of gene products across all species, and REACTOME, a tool that identifies biological pathways that are enriched in a gene list, to predict function of the proteins whose levels were significantly different (+/−50%) (Thomas, 2003; Jassal et al., 2019). The GO analysis revealed that, in both groups, the predominant protein class was metabolite interconversion enzymes, followed by nucleic acid-binding proteins. This prediction indicated that the majority of proteins with high and low spectral counts in both cell lines played catalytic or protein-binding roles. The results for the biological process shown in Figure 3.4 corroborates the similarity between the two groups observed in the protein class and molecular functional analyses, with most proteins being implicated in different metabolic or general cellular processes. For a thorough understanding of the cellular implications of Cul3 absence, I have also explored the REACTOME biological pathway database to identify potential pathways that were altered in Cul3 KO cells (Tables 3.3 and 3.4). The analysis revealed that most proteins with high and low spectral counts were involved in
nucleic-acid processing, metabolism, or signal transduction processes, which was in accordance with the results of the GO enrichment analysis (Figure 3.5).

**PROTEINS WITH SIGNIFICANT HIGH SPECTRAL COUNTS IN CUL3 KO CELLS**

**Identification of potential substrates and pathways that contribute to a more severe FHHt phenotype observed in Cul3 knockouts:** Previously characterized substrates of Cul3, such as WNK1, RhoA, and RhoB, as well as the Cul3 substrate adaptor ANKFY1 (Table 3.5; Terker et al., 2018; Cornelius et al., 2018; Chen et al., 2009; Murakami et al., 2019; Maekawa et al., 2017) shown high spectral counts in our MS-based proteomic analysis, confirming the reliability of the procedure, and supporting our strategy of using KO cells to discover novel Cul3 targets, substrate adaptors, and pathways. The analysis also revealed a subset of proteins with high spectral counts in the KO cells that have not yet been characterized as Cul3 substrates but have the potential to be (Table 3.6). To further analyze these proteins, I categorized them based on the three criteria: First, using information from the literature, I identified where they are expressed and active in the kidney, and checked if they overlapped with Cul3 expression and activity. Second, I determined the renal pathways of involvement and predicted if disruptions and mutations in these pathways could collaborate with the severity of the FHHt phenotype. Lastly, I searched the literature for information connecting them to Cul3...
function, which would support our hypothesis that they are potential Cul3 substrates. The analysis is described below.

Mitogen-activated protein kinases (MAPKs)

The first of the proteins that meet the three criteria listed above are two mitogen-activated protein kinases (MAPKs), known as ERK1 and ERK2. Similar to Cul3, these two enzymes are highly expressed and active in the distal convoluted tubules (DCT; Cornelius et al., 2019; Capolongo et al., 2019). This renal colocalization indicates a possibility of physical interaction between them, which is consistent with them having the potential to be Cul3 substrates. ERK1 and ERK2 are known to mediate cellular responses to injuries and stress in the kidneys as well as the regulation of the thiazide-sensitive NCC (Ko et al., 2010).

As previously mentioned, upregulation of NCCs is the main cause of the increased blood pressure and elevation of potassium levels observed in FHHt patients that are triggered by disruption of Cul3 and, as a result, enhanced activity of its substrates WNK1 and WNK4 (McCormick et al., 2014). The fact that both WNKs and ERKs are kinases and control the activity of NCCs in the DCT supports the hypothesis that the severe phenotype observed in Cul3 knockouts is caused by simultaneous enhanced activity of multiple substrates, and the ERKs are possibly part of this regulatory pathway. In addition, Hollstein & Cichowski has shown that Cul3 loss, drastically attenuated ERK signaling in a study about the regulation of the NF1 tumor suppressor protein by the BCR complex (Hollstein & Cichowski,
Taken together, these results support our hypothesis that ERK1 and ERK2 are strong candidates for Cul3 substrates and, in addition to WNK1 and WNK4, they are likely to regulate the activity of the NCCs. Further studies would be required to properly understand their roles in the cell.

**Syntaxin 4A (STX4A)**

Second, I analyzed the vesicle-targeting protein Syntaxin 4a (STX4A), which, like Cul3, is also expressed in the principal cells of the renal collecting ducts (CD). Compared to the cytoplasmic Cul3, STX4A is an integral membrane protein localized to the apical plasma membrane of the CD cells (Cornelius et al., 2019; Mandon et al., 1996). Regarding its cellular role, STX4A is known to target Aquaporin 2 (AQP2)–containing vesicles to the apical plasma membrane (Mandon et al., 1997). Apical plasma membrane faces urine containing lumen of CD. As previously demonstrated by McCormick, AQP2 levels are reduced in Cul3 knockout mice (McCormick et al., 2014). A possible explanation for this phenomenon is that, in the absence of Cul3, the expression of RhoA increases. RhoA, a known Cul3 substrate and a regulator of the cytoskeleton, could prevent the association of AQP2-containing vesicles with the apical membrane of the DC cells, which in turn causes disposal of AQP2 in the urine.

My analysis showed that the spectral counts of RhoA were two times higher in the KO cells while AQP2 levels were zero, supporting the hypothesis that, in the absence of Cul3, the enhancement of RhoA probably affects the levels of AQP2.
RhoA is a well-known Cul3 substrate, STX4A and RhoA have antagonistic roles in the kidney, and the absence of protein-protein interactions between Cul3 and STX4A (Rouillard et al., 2016) suggests that STX4A is likely not a Cul3 substrate. In addition, a study about Syntaxin 1 demonstrated that a novel E3 ubiquitin-protein ligase known as Staring was responsible for targeting Syntaxin 1 for degradation (Chin et al., 2002), supporting the idea that Cul3 is probably not involved in the degradation of members of the Syntaxin family. However, we and many others have shown that multiple E3 ligases target substrates, so it is possible that STX4A is in fact a substrate.

**Gelsolin (GSN)**

Thirdly, I analyzed Gelsolin, which is an actin-modulating protein responsible for promoting actin filament nucleation, as well as severing existing filaments (Hartwig et al., 1990). Similar to Cul3, it is expressed in the distal convoluted tubules (DCTs; Cornelius et al., 2019; Lueck et al., 1998; Arai & Kwiatkowski, 1998), which meets the first criteria to be considered a potential Cul3 substrate. In the kidney, GSN functions as part of the extracellular actin-scavenging system, binding and facilitating removal of potentially inflammatory actins released from injured cells (Shi et al., 2018). The symptoms observed in FHHt are known to cause severe damage to the kidney cells, which presumably triggers the release of several inflammatory actins. In response, GSN is activated, and its scavenging process begins. If GSN was a Cul3 substrate, in a scenario of
FHHt diagnosis, we would observe an enhancement in GSN levels due to
malfunction in the Cul3. Increase in GSN would lead to a reduction in
proinflammatory actins, and an overall improvement in the kidneys' health. It is the
opposite of what is observed in FHHt. Interestingly, I observe GSN’s 2.6X fold
change, which suggests that Cul3 controls GSN’s cellular levels, either directly or
possibly through the degradation of other yet to be discovered proteins responsible
for GSN degradation. Further analysis will be necessary to identify them.

*Other potential Cul3 substrates with less clear associations*

I also found two other proteins that are not associated with FHHt but have
the potential to be Cul3 substrates (Table 3.7). The first one is a tumor suppressor
known as BCCIP (BRCA2 and CDKN1A-interacting protein), which is an important
cofactor for BRCA2 in tumor suppression, and a modulator of CDK2 kinase activity
via p21 (Liu et al., 2001). BCCIP is involved in important processes relevant to the
maintenance of genome stability and its downregulation is correlated with the
aggressiveness of brain tumors (Liu et al., 2009). The second one is another tumor
suppressor known as RB1CC1 (Rb1-inducible coiled-coil protein 1), which is a
potent regulator of the RB1 pathway through induction of RB1 (Retinoblastoma 1)
expression (Chano et al., 2002). RB1CC1 is involved in multiple processes such
as cell growth, cell proliferation, apoptosis, autophagy, and cell migration, and its
dysregulation is associated with cell growth and progression of various cancers
(Kontani et al., 2003). Co-immunoprecipitation assays of these two proteins may
reveal them as promising targets for future studies investigating the role of the Cul3-based ubiquitin ligase in FHHt development and other pathological processes.

**Common domains found among the proteins with significant high spectral counts:** I further characterized the relationship between Cul3 and the proteins that shown a high spectral count in the analysis by searching for a common pattern of conserved domains among them. By isolating regions that are conserved in the proteins with high spectral counts, I wanted to identify common elements that could be valuable for further functional studies about how Cul3 interacts with other proteins and for evolutionary studies about how these interactions evolved to fulfil specialized cellular functions. For instance, previous studies have discovered members of cullin complexes through their protein-protein interacting domains, such as F-box proteins in the Cul1 complex, BTB domain-containing proteins in the Cul3 complex, and DWD proteins in the Cul4A complex (Bai et al., 1996; Skowyra et al., 1997; Furukawa et al., 2003; He et al., 2006). Furthermore, Cullin-RING ligases (CRLs) are structurally similar, but there is almost no overlapping regarding the substrates they ubiquitinate and the structural domains their substrate adaptors use to connect the substrates to the CRL (Sun et al., 2020). Here, I identified four domains of interest that will be used in further analyzes: Ubiquitin-specific protease (UPS) domain, UBC fold domain, leucine-rich repeat (LRR) domain, and WD40 domain (Table 3.8).


Ubiquitin-specific proteases (USPs)

Ubiquitin-specific proteases are deubiquitinating enzymes (DUB), which are proteins responsible for removing ubiquitin from modified proteins, recycling ubiquitin attached to inappropriate targets, disassembling polyubiquitin chains, and processing proteins before their degradation by the proteasome (Sippl et al., 2011). The USP domain consists of a common conserved catalytic core interspersed at five different points with insertions, some of which are as large as the catalytic domain itself. These insertions can fold into independent domains that can be involved in regulating the DUB activity (Ye et al., 2009). The 14 DUB identified in the KO cells suggests that Cul3 plays an important role in their expression. There are two possible explanations for this: (1) They might be substrates of Cul3, so in its absence, their levels increase because they are not ubiquitinated and targeted for degradation. (2) Cul3 role is broader than targeting substrates to degradation by the proteasome. In fact, a huge number of its substrates are involved in regulatory responses or signal transduction pathways. Therefore, it is possible that the lack of Cul3 halts the ubiquitination of regulatory molecules involved in controlling the expression and/or degradation of DUB enzymes. Biochemical verification of the 14 USP identified in this analysis may help to understand the mechanisms behind their overexpression and may reveal promising candidates for future studies investigating the role of the Cul3-based ubiquitin ligase.
**UBC fold domain-containing proteins**

Members of the family of ubiquitin-conjugating enzymes (E2s) are characterized by the presence of a highly conserved ubiquitin-conjugating catalytic (UBC) fold. Some E2s consist only of the catalytic domain (class I), others have additional N- or C-terminus extensions (classes II and III, respectively) or both (class IV) (Figure 1.5; Valimberti et al., 2015). These extensions are involved in functional differences between E2s, which involve differences in subcellular localization, stabilization of the interaction with E1 enzymes, or modulation of the activity of the interacting E3 ligase (Durfee et al., 2008; Clague et al., 2015; Eletr et al., 2005). Fifteen different ubiquitin-conjugating enzymes were identified in our analysis (Table 3.8). Initially, we were inclined to characterize them as partners of Cul3 during the transference of ubiquitin to the substrates, but the data shown in Figure 2.6 revealed that a few of these E2s did not interact with Cul3. Alternatively, another possibility is that these E2s are not serving as a ubiquitin-conjugating enzyme with Cul3 but are instead bound to Cul3 as substrates. Biochemical verification of these enzymes will help us to expand our knowledge about the preferential binding of E2s for specific cullins and may reveal promising candidates for future studies about the major players in the process of ubiquitin transference to the substrates associated with FHHt and other disorders.
**Leucine-rich repeat (LRR) proteins**

LRR domains are composed of repeating 20–30 amino acid stretches that are unusually rich in the hydrophobic amino acid leucine (Kobe & Deisenhofer, 1994). They are composed of tandem copies of LRR repeat (also known as α/β horseshoe fold) that typically fold together to form a solenoid protein domain very similar to the one observed in the WD40 domain-containing proteins. The LRR repeat is present in many prokaryotic and eukaryotic proteins and is one of the most commonly occurring protein domains in proteins associated with innate immunity (Ng et al., 2011). Leucine-rich repeats are frequently involved in the formation of protein-protein interactions, so might as well interact with Cul3 and/or with its substrates.

**WD40 domain-containing proteins**

WD40 domains are β-propeller domains that act as protein interaction scaffold in multiprotein complexes (Stirnimann et al., 2010). They are composed of tandem copies of the WD40 repeat (also known as the WD or beta-transducin repeat) that typically fold together to form a type of circular solenoid protein domain (Xu & Min, 2011). They are among the most abundant identifiable protein domains (Schapira et al., 2017). Found in a wide variety of eukaryotic proteins, WD40 domain-containing proteins perform functions such as adaptor/regulatory modules in a diverse array of cellular processes (Xu & Min, 2011). Their ability to act as a
scaffold platform for a stable or reversible association of binding partners may be useful for the Cul3 complex assembling and/or interaction with its substrates.

**Potential substrate adaptors for the Cul3 complex:** In 2014, our group identified a new set of Cul3-bound proteins that contained either the LRR or WD40 domains and were known as CLWs (Wimuttisuk et al., 2014). We also demonstrated that the LRR domain-containing proteins could bind both Cul3 and BTB domain-containing proteins. This dual binding role for the LRRs causes the BTB-domain protein to become a substrate instead of an adaptor. To our surprise, our MS-based proteomic study identified 32 WD40 domain-containing proteins and 9 LRR domain-containing proteins (Table 3.8). At the initial stage of this data analysis, we were inclined to designate these proteins as potential substrates for the Cul3-based ubiquitin ligase complex. However, after a careful functional comparison, we found some compelling evidence supporting the hypothesis that they may be Cul3 substrate adaptors, not substrates. (1) As previously mentioned, we have observed increased abundance of two Cul3 adaptors, Klhl3 and Keap1, in cells lacking Cul3 (McCormick et al., 2014; Watai et al., 2007). (2) Besides the dual binding role of LRR domain-containing proteins characterized by Wimuttisuk et al., crystal structures of other cullin complexes revealed that the WD40 and the LRR domains are also common among F-box and DWD proteins, which serve as substrate adaptors for the SCF (Skp1-Cul1-F-box) ubiquitin ligase complex and the DDB1-Cul4A ubiquitin ligase complex. (Zheng et al., 2002; Angers et al.,
2006a; He et al., 2006). Taken together, I propose an alternative model for the WD40/LRR domain-containing proteins as novel substrate adaptors of the BCR ubiquitin ligase complex, instead of investigating them as potential substrates. Future biochemical analysis will be essential to define the cellular role of these two subsets of proteins.

PROTEINS WITH SIGNIFICANT LOW SPECTRAL COUNTS IN CUL3 KO CELLS

We have previously shown that Cul3 KO cells have elevated levels of several known Cul3 substrates (McEvoy et al., 2007; Davidge et al., 2019). Therefore, in order to identify candidate Cul3 targets and pathways that may contribute to the initial renal injury observed in KS-CUL3 KO mice, we primarily focused our analysis on the proteins with high spectral counts. However, I observed that among the 3265 proteins identified in both cell lines, 317 had a fold change ≤ -1.5, with 46 proteins showing a statistically significant difference between WT and Cul3 KO cells (p-value ≤ 0.05). I hypothesize that this phenomenon is caused by an enhancement in levels of intermediary proteins that regulate the expression of the ones with reduced levels in Cul3 KO cells compared to WT. These intermediary regulators are likely to be ubiquitinated by Cul3 and targeted to degradation by the 26S proteasome. However, in Cul3 KO cells, they can no longer be ubiquitinated, which leads to a rise in their cellular levels and, consequently, a reduction in the levels of their target proteins (Figure 3.6). Further
tests will be necessary to test this hypothesis and, hopefully, they will help to expand our understanding of all the cellular pathways in which Cul3 is involved.

Validation of the proteomics data by immunoblot analysis: To validate the proteomics data, we focused our attention on two proteins of interest, whose spectral counts were altered and for which antibodies were available. These were ubiquitin-conjugating enzyme D1 (UbE2D1) and Leucine-rich repeat 1 (LRR1). As shown in Figure 3.7A, the levels of UbE2D1 undergo dramatic alteration in the KO in comparison to the WT cells. The same applies to LRR, whose amount is lower in the absence of Cul3 while being unaffected or even slightly increased in the WT cells (Figure 3.7B). The variations observed in the Western blot analysis are in accordance with the data obtained in the Mass Spectrometry analysis and reveal a distinguishable effect on the proteome of KO compared to the WT cells.

DISCUSSION

The discovery of Cul3 launched a rapidly expanding research niche that had a strong potential to affect clinical practice. The characterization of this E3 ligase has evolved quickly and resulted in the identification of a variety of substrates and members of the BCR complex (Xu et al., 2003; Pintard et al., 2004; Dubiel et al., 2017). Yet, there is still much to be discovered to fully understand the nature of Cul3 protein. In the work presented here, MS-based proteomics identified novel components of the Cul3 complex and possible pathways of Cul3 interactions. I
categorized potential members of the Cul3 complex using three different approaches: (1) classification of potential Cul3-binding partners based on their molecular function and class, (2) identification of potential substrates and cellular processes, and (3) determination of the conserved domains of the proteins with high spectral counts and their roles in the Cul3-based ubiquitin ligase complex. Here, I discuss the significance of my findings, present an alternative BCR complex model based on the analysis of conserved domains, and consider future studies that may arise from this MS-based proteomics experiment.

**Pathways in which Cul3 is involved:** Prior studies have identified multiple abnormalities in cells that lack Cul3 (Genschik et al., 2013; Dong et al., 2020; Cornelius et al., 2018). Here, I have extended these past approaches and demonstrated more defined alterations of multiple cytoskeletal, translational, and developmental proteins in Cul3 KO cells (Figure 3.4). Changes in the three protein categories are consistent with the reported biology of Cul3 (Figure 3.5A and 3.5B). For example, Cul3 was shown to mediate the transport and biogenesis of vesicles in the Golgi apparatus, the secretion and concentration of large cargo in the ER, and the synthesis of IPs in the ER lumen (McGourty et al., 2016; Jin et al., 2012; Saito & Katada, 2015; Kim et al., 2018; Bisnett et al., 2021). Similarly, Cul3 was also shown to regulate the levels of proteins involved in DNA damage response and repair, cell proliferation, and development of malignancies (Ribar et al., 2007; Sun et al., 2016; Vanneste et al., 2020). Overall, the broad range of proteins that
were differentially regulated in my proteomics analysis confirmed that endogenous Cul3 is associated with several signaling pathways and affects proteins in three major categories.

**Role of Cul3 in Familial Hyperkalemic Hypertension:** Arterial hypertension is the number one risk factor for death worldwide (Narayan et al., 2010), with a prevalence ranging between 30 and 40% in the adult population. Familial hyperkalemic hypertension (FHHt), also known as Gordon syndrome or pseudohypoaldosteronism type II, is a rare inherited hypertension syndrome (McCormick et al., 2014). It is characterized by severe arterial hypertension, hyperkalemia, hyperchloremic metabolic acidosis, hypercalciuria, and low renin state. Cul3, Klhl3, Wnk1, and Wnk4 are named as the four foes causing FHHt. A remarkable body of work aimed at understanding how the product of these genes modulates ion transport in the distal part of the nephron and, thus, causes hypertensive manifestations (Farfel et al., 2019). Mutations in Klhl3 produce a dominant or recessive form of FHHt (Boyden et al., 2012; Louis-Dit-Picard et al., 2012), and mutations in Cul3 are associated with an increased severity of FHHt (Boyden et al., 2012). The Cul3-Klhl3 complex recruits the WNK kinases for ubiquitination to promote their proteasomal degradation. Mutations in Klhl3 disrupt the interaction with the substrates or Cul3, thus preventing the degradation of WNKs (McCormick et al, 2014). Mutations in Cul3 also abrogate WNK ubiquitination, but the effect is indirect, via increased ubiquitination and
degradation of KLHL3 (Ohta et al., 2013). Besides Klhl3, Wnk1, and Wnk4, several other proteins have shown to be affected by mutations of Cul3 and, consequently, are also involved in the development of FHHt. Our functional analysis has revealed that Wnk1, RhoA, and RhoB were upregulated in the KO cells, which is in accordance with the results described in the literature (Terker et al., 2018; Cornelius et al., 2018; Chen et al., 2009; Murakami et al., 2019;). In addition, our analysis has also identified four other proteins that were upregulated in the KO cells and play specific physiological roles in the kidney. Even though they have not been characterized as Cul3 substrates yet, ERK1 and ERK2 have the potential to be ubiquitinated by Cul3 and, possibly, be involved in the severe phenotype of FHHt.

**Model of the novel BCR\textsuperscript{WD40/LRR} complex:** A conserved domain analysis revealed domains that are frequently found in proteins that interact with Cul3. In particular, we observed that proteins containing the WD40 and the LRR domains are present more often in the wild type than in the knockout cells. Initially, we hypothesized that these proteins represented groups of substrates that were recognized by the Cul3 complex via their conserved domains. However, a structural analysis revealed that the WD40 and the LRR domains were the substrate adaptor domains of the F-box proteins in the Cul1-based ubiquitin ligase complex (Schulman et al., 2000; Zheng et al., 2002). Furthermore, the similarity between the Skp1 and BTB domains also suggests that the binding between BTB domain-containing proteins
and WD40/LRR domain-containing proteins may be similar to the binding interaction between the Skp1 and F-box proteins (Pintard et al., 2004). Additionally, a study using MudPIT analysis of the Cul4A-based ubiquitin ligase complex has identified clusters of WD40 domain-containing proteins that were later shown to be substrate adaptors for the Cul4A complex (Angers et al., 2006a; He et al., 2006). Therefore, I propose that the WD40/LRR domain-containing proteins are likely to be substrate adaptors for the Cul3 complex, not substrates.

**Future studies:** In conclusion, our MS-based proteomic analysis revealed a substantial amount of information that can be used to characterize the Cul3-based E3 ligase complex in future experiments. First, I have identified two potential Cul3 substrates involved in FHHt and two involved in cancer. Second, I have found clusters of proteins that contain UPS, UBC fold, WD40 or LRR domains, which suggests they may be used for protein-protein interaction with Cul3, its substrate adaptors, or its substrates. Third, I proposed that these WD40 and LRR proteins are likely to serve as additional substrate adaptor modules for the Cul3-based ubiquitin ligase complex when their BTB domain-containing proteins cannot recruit substrates due to the absence of a second protein-protein interacting domain. Although additional biochemical experiments are required to confirm the proposed models, future characterization of the Cul3 complex based on these hypotheses will lead to a more thorough understanding of the ubiquitination mechanism of the Cul3-based ubiquitin ligase complex and its correlation to other cellular processes.
MATERIALS AND METHODS

Cell culture and transfections: Cells (HEK293 wild type and Cul3 knockout) were maintained in DMEM (Gibco™, CAT no. 11965092) supplemented with 10% Fetal Bovine Serum (Atlas Biological, CAT no. F-0500-A), 1% L-Glutamine (Gibco™, CAT no. 25030081), and 1% Penicillin/Streptomycin (Gibco™, CAT no. 15070063). Cells were split 1:20 for transfection in 6 cm dishes the night before transfection. Transfections were performed using the calcium phosphate precipitation (Jordan et al., 1996). For immunoprecipitations and Western blots, cells were harvested 48 hours post-transfection. The amount of plasmid DNA transfected into each plate varied from 1 to 10μg. All transfected cells were scrapped and harvested using 600μL of a solution containing 10mL of radioimmunoprecipitation assay lysis buffer (RIPA) mixed with 1 protease inhibitor tablet (Thermo Scientific™, CAT no. PIA32953), and then sonicated with an ultrasonic cell disrupter (VIRTIS VIRSONIC 100) for 15 seconds at 50% power before being used for immunoprecipitations or western blots. CRISPR knockout Cul3 HEK293 cells were a gift from Curt Sigmund.

Western blotting and immunoprecipitations: Western blots and immunoprecipitations were conducted as previously described (Wimuttisuk et al., 2014). In short, 500μL of the sonicated transfection lysate was added to the desired antibody in a microcentrifuge tube. 40 μl of RIPA–Sepharose beads (Thermo Scientific™, CAT no. 101041) were then added to the mixture, and the immunoprecipitations were placed on a rotator for 2 h at room temperature before
being rinsed with RIPA buffer for 15 seconds at 1500rpm, heated in 15µL of SDS-PAGE loading buffer for 2 minutes, and ran on an SDS-PAGE gel. The following antibodies were used for immunoprecipitations at 1–2 μg or at the indicated dilution for western blotting: polyclonal anti-HA (1:1000, CAT no. PA1-985, Invitrogen™), polyclonal anti-β actin (CAT no. AM4302, Invitrogen™), polyclonal anti-Cul3 (Singer et al., 1999; McEvoy et al., 2007), and monoclonal anti-cyclin E (HE12) (1:100 for transfected samples, CAT no. 32-160-0, Invitrogen™), Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (1:10000 for transfected samples, CAT no. 31460, Invitrogen™), Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (1:10000 for transfected samples, CAT no. G-21040, Invitrogen™). Western blots were visualized using myECL™ Imager (CAT no. 62236, Thermo Scientific™).

Peptide preparation, desalting, and reconstitution: Cell lysates were collected as described above, and their protein concentrations were measured with a BCA assay (CAT no. 23225, Thermo Scientific™). Extracted proteins were reduced with 45 mM DTT at 60 °C for 20 min, and alkylated with 100 mM IAA in the dark at room temperature for 15 min. The proteins were digested with trypsin (Promega, CAT no. V5111) at a ratio of 1:50 (trypsin/protein w/w) at 37 °C overnight. Digested peptides were desalted with a SepPak C18 cartridge (Waters, CAT no. WAT051910), kept in −80 °C for 1h, and dried in a lyophilizer for 48h. Then, the digested peptides were stored at −80°C. Dry peptides were centrifuged for 5 min at room temperature and resuspended in 0.9X of the total volume using 0.1M
acetic acid. The peptide suspensions were briefly centrifuged to collect the resuspensions at the bottom of the tubes then transferred clear 1.5 mL tubes. The samples were centrifuged at 13000x for 5 min at room temperature, and 9uL of peptide supernatant was added to new tubes containing 1uL or peptide standards. The tubes were sealed and briefly centrifuged to remove bubbles and collect the samples at the tubes' bottom.

**Automated MS-based label-free proteomics analysis:** The mass spectrometry and protein sequence prediction were performed by a collaborator from the Salomon lab - Brown University.

**Data analysis and peptide quantification:** Data analysis was primarily performed using GraphPad Prism 9, MaxQuant, and Microsoft Excel. The differences between the two groups were analyzed with a two-tailed Students t-test.

**Gene Ontology (GO) enrichment analysis:** The PANTHER version 15.9 Online Tool was used to perform a functional GO enrichment analysis on the two proteomics datasets, WT and Cul3 KO. Step 1: the UniProt IDs of the proteins with significant high or low spectral counts were entered in the Panther software interface for a comparison to its reference ID list. Step 2: the organism selected for comparison was *Homo sapiens*. Step 3: the analysis selected was “Functional classification viewed in graphic charts” followed by the selection of the option was “Pie-charts”.

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**REACTOME analysis:** The “Analyze gene list” was the Reactome tool chosen for this study. Step 1: the UniProt IDs of the proteins with significant high or low spectral counts were entered in the Reactome software interface for a comparison to its reference ID list. Step 2: the preferred option selected was “Include interactors”. Step 3: the visualization mode selected was “Voronoï”.
FIGURES

**Figure 3.1** – **Validation and characterization of Cul3 KO cells:** A) Cellular lysates of two replicates of wild type (WT) and two replicates of CUL3 knockout (KO) clones were analyzed by western blot with the indicated antibodies. Actin is the loading control. B) Comparison of cyclin E expression levels between WT and KO clones. C) WT cells and KO clones were treated with cycloheximide (CHX) for 12h, and cyclin E half-life was determined. Actin is the loading control. This experiment was repeated three times and yielded similar results.
Table 3.1 - Reproducibility of LC-MS/MS results: Number of proteins observed in four WT and four Cul3(-/-) biological replicates.

<table>
<thead>
<tr>
<th>Biological Replicate</th>
<th>WT 1</th>
<th>WT 2</th>
<th>WT 3</th>
<th>WT 4</th>
<th>KO 1</th>
<th>KO 2</th>
<th>KO 3</th>
<th>KO 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Proteins</td>
<td>2869</td>
<td>2875</td>
<td>2847</td>
<td>2737</td>
<td>2896</td>
<td>3018</td>
<td>2687</td>
<td>2532</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>2832 ± 56</td>
<td></td>
<td></td>
<td>2783 ± 187</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9178</td>
</tr>
</tbody>
</table>

**Biological Replicates:** Four biological replicates of WT and KO cell lines were used in the LC-MS/MS analysis. The replicates referred in this work are biological replicates, not analytical or technical replicates of a single biological sample.

**Proteins:** The relative amount of a specific protein was calculated using the number of observed spectral count of the specific protein and the internal standards.

**Statistical Analysis:** Student t-test was used to compare the number of unique peptides identified in the four WT replicates and four KO replicates. The t-test was performed with two-tailed in which the number of proteins of the four WT replicates were compared to the number of proteins of the four KO replicates. The hypothesis was that there were significant differences between the replicates and statistically this was refuted (p-value of 0.9178).
Table 3.2 - Reproducibility of LC-MS/MS results: Number of unique peptides observed in four WT and four Cul3(-/-) biological replicates.

<table>
<thead>
<tr>
<th>Biological Replicate</th>
<th>WT 1</th>
<th>WT 2</th>
<th>WT 3</th>
<th>WT 4</th>
<th>KO 1</th>
<th>KO 2</th>
<th>KO 3</th>
<th>KO 4</th>
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</thead>
<tbody>
<tr>
<td>Number of Unique Peptides</td>
<td>13748</td>
<td>14007</td>
<td>13479</td>
<td>12279</td>
<td>14400</td>
<td>14878</td>
<td>13420</td>
<td>11203</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>13378 ± 662</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.6807</td>
</tr>
</tbody>
</table>

**Biological Replicates**: Four biological replicates of WT and KO cell lines were used in the LC-MS/MS analysis. The replicates referred in this work are biological replicates, not analytical or technical replicates of a single biological sample.

**Unique Peptides**: A unique peptide is defined as a peptide, irrespective of its length, that exists only in one protein of a proteome of interest, even though this peptide may appear more than once in the same protein.

**Statistical Analysis**: Student t-test was used to compare the number of unique peptides identified in the four WT replicates and four KO replicates. The t-test was performed with two-tailed in which the number of unique peptides of the four WT replicates were compared to the number of unique peptides of the four KO replicates. The hypothesis was that there were significant differences between the replicates and statistically this was refuted (p-value of 0.6807).
Figure 3.2 – Workflow of the mass spectrometry-based label-free quantitative proteomics: WT and KO samples were subjected to individual LC-MS/MS analysis and the quantification was based on the comparison of peak intensity of the same peptide or the spectral count of the same protein.
### A

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Protein groups</td>
<td>4087</td>
</tr>
<tr>
<td>Protein groups common to both cell lines</td>
<td>3265</td>
</tr>
<tr>
<td>Altered proteins KO vs WT (p-value (\leq 0.05))</td>
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<tr>
<td>Altered proteins KO vs WT (p-value (\leq 0.05) and Fold Change (\geq 1.5))</td>
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</tr>
<tr>
<td>Altered proteins KO vs WT (p-value (\leq 0.05) and Fold Change (\leq 1.5))</td>
<td>46</td>
</tr>
</tbody>
</table>

### B

**Volcano plot analysis**

- 3265 protein groups observed in both cell lines
- 123 significant proteins
- 77 proteins (p-value \(\leq 0.05\) and Fold Change \(\geq 1.5\))
- 46 proteins (p-value \(\leq 0.05\) and Fold Change \(\leq 1.5\))
Proteins with higher spectral counts in the Cul3 KO cells than in WT cells

- Serine hydroxymethyl transferase, mitochondrial
- EBNA2 coactivator p100
- Protein disulfide isomerase
- Solute carrier family 3, member 2
- Complement component C1q binding protein
- Myosin heavy chain, nonmuscle type B
- ORP150
- ATP citrate lyase
- DnaJ homolog subfamily C member 7
- Prolyl endopeptidase
- HIV 1 rev binding protein 2
- NAD(P)H steroid dehydrogenase like protein
- Chromosome 16 open reading frame 34
- Acyl-CoA thioesterase 7
- Plastin 1
- Basic transcription factor 3
- Peroxisomal acyl-coenzyme A thioester hydrolase 2a
- LOC63929
- DnaJB11
- Valyl tRNA synthetase 2
- Chromosome 1 open reading frame 33
- ATP binding cassette, sub family F, member 2
- RING3
- Pyrroline-5-carboxylate reductase 1
- Nodal modulator 1
- Insulin receptor substrate 4
- HEAT repeat containing 3
- Splicing factor 3A, subunit 2
- Prolyl 3-hydroxylase 1
- WD repeat domain 6
- Erythrocyte membrane protein band 4.1
- Glucose-6-phosphate dehydrogenase
- A kinase PRKA anchor protein 8 like
- Chromosome 1 open reading frame 69
- Ribosomal protein L34
- A kinase PRKA anchor protein 8 like
- Glutathione peroxidase 4
- SNX5
- Epsin 4
- Elongation factor 1, alpha 2
- Galactosidase, alpha
- ATPase H+ transporting lysosomal noncatalytic accessory protein 1A
- Plectin 1
- Lysine hydroxylase
- Argininosuccinate synthetase
- Ubiquitin conjugating enzyme EZD1
- Transcription factor IIF, alpha subunit
- Rab-coupling protein
- MTATP6
- KIAA1495
- Golgi specific brefeldin A resistance factor 1
- Copine II
- Signal sequence receptor alpha
- Lanosterol synthase
- Surfeit 4
- Protease inhibitor 6
- KIAA0664
- TLOC1
- WD repeat domain 44
- SNF1 sucrose nonfermenting like kinase
- Legumain
- Carbonic anhydrase VIII
- ELAC
- ANKFY1
Figure 3.3 – Proteomic analysis of WT and KO cells: (A) Summary of the results observed in the LC-MS/MS proteomics analysis. (B) Volcano plot of the 4087 proteins quantified in the four WT and four Cul3 KO replicates. (C) KO/WT ratio of the proteins that a significant difference in their spectral counts. WT refers to wild type, and KO refers to Cul3 knockout replicates.
Figure 3.4 – Functional analysis of proteins with significant high and low spectral counts in Cul3 KO cells: (A) PANTHER (Protein ANalysis THrough Evolutionary Relationships) of statistically significant downregulated and (B) upregulated proteins in CUL3 KO cells.
Figure 3.5 - REACTOME pathway analysis of proteins whose spectral counts were significantly different (+/-50%): Figures (A) Upregulated and (B) Downregulated show a genome-wide overview of the results of the REACTOME pathway analysis. REACTOME pathways are arranged by hierarchy. The color code denotes over-representation of that pathway in the input dataset. Light gray signifies pathways which are not significantly over-represented.
Table 3.3 – The 25 most relevant pathways that are upregulated in Cul3−/−/− cells sorted by p-value. FDR refers to False Discovery Rate. (Source: REACTOME).

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Entities</th>
<th>Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>found</td>
<td>ratio</td>
</tr>
<tr>
<td>Organic anion transporters</td>
<td>3 / 10</td>
<td>8.74e-04</td>
</tr>
<tr>
<td>RHO GTPases activate CIT</td>
<td>4 / 20</td>
<td>0.002</td>
</tr>
<tr>
<td>Activation of gene expression by SREBP (SREBP)</td>
<td>6 / 43</td>
<td>0.014</td>
</tr>
<tr>
<td>trans-Golgi Network Vesicle Budding</td>
<td>8 / 74</td>
<td>0.006</td>
</tr>
<tr>
<td>Mitochondrial calcium ion transport</td>
<td>4 / 23</td>
<td>0.002</td>
</tr>
<tr>
<td>GDP-fucose biosynthesis</td>
<td>2 / 6</td>
<td>5.25e-04</td>
</tr>
<tr>
<td>Cholesterol biosynthesis</td>
<td>4 / 26</td>
<td>0.002</td>
</tr>
<tr>
<td>mRNA decay by 5' to 3' exoribonuclease</td>
<td>3 / 15</td>
<td>0.001</td>
</tr>
<tr>
<td>Golgi Associated Vesicle Biogenesis</td>
<td>6 / 56</td>
<td>0.005</td>
</tr>
<tr>
<td>Deadenylation-dependent mRNA decay</td>
<td>6 / 56</td>
<td>0.005</td>
</tr>
<tr>
<td>Regulation of cholesterol biosynthesis by SREBP (SREBP)</td>
<td>6 / 56</td>
<td>0.005</td>
</tr>
<tr>
<td>EPHA-mediated growth cone collapse</td>
<td>4 / 29</td>
<td>0.003</td>
</tr>
<tr>
<td>Defective Base Excision Repair Associated with NTHL1</td>
<td>1 / 1</td>
<td>8.74e-05</td>
</tr>
<tr>
<td>Defective NTHL1 substrate binding</td>
<td>1 / 1</td>
<td>8.74e-05</td>
</tr>
<tr>
<td>Synthesis of IPs in the ER lumen</td>
<td>1 / 1</td>
<td>8.74e-05</td>
</tr>
<tr>
<td>Defective NTHL1 substrate processing</td>
<td>1 / 1</td>
<td>8.74e-05</td>
</tr>
<tr>
<td>Defective TPMT causes Thiorpurine S-methyltransferase deficiency (TPMT deficiency)</td>
<td>1 / 1</td>
<td>8.74e-05</td>
</tr>
<tr>
<td>tRNA modification in the nucleus and cytosol</td>
<td>5 / 43</td>
<td>0.004</td>
</tr>
<tr>
<td>RHO GTPases Activate ROCKs</td>
<td>3 / 19</td>
<td>0.002</td>
</tr>
<tr>
<td>Sema4D induced cell migration and growth-cone collapse</td>
<td>3 / 20</td>
<td>0.002</td>
</tr>
<tr>
<td>Beta-oxidation of pristanoyl-CoA</td>
<td>2 / 9</td>
<td>8.74e-04</td>
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<tr>
<td>RMTs methylate histone arginines</td>
<td>5 / 49</td>
<td>0.004</td>
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<tr>
<td>Signal amplification</td>
<td>4 / 35</td>
<td>0.003</td>
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<tr>
<td>Displacement of DNA glycosylase by APEX1</td>
<td>2 / 10</td>
<td>8.74e-04</td>
</tr>
<tr>
<td>Glycogen storage disease type 0 (liver GYS2)</td>
<td>1 / 2</td>
<td>1.75e-04</td>
</tr>
</tbody>
</table>
Table 3.4 – The 25 most relevant pathways that are downregulated in Cul3(-/−) cells sorted by p-value. FDR refers to False Discovery Rate. (Source: REACTOME).

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>Entities found</th>
<th>Entities ratio</th>
<th>p-value</th>
<th>FDR*</th>
<th>Entities found</th>
<th>Entities ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cargo concentration in the ER</td>
<td>4 / 59</td>
<td>0.663</td>
<td>0.014</td>
<td>0.578</td>
<td>7 / 12</td>
<td>0.076</td>
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<tr>
<td>Erythropoietin activation STAT3</td>
<td>2 / 7</td>
<td>6.12e-94</td>
<td>0.017</td>
<td>0.578</td>
<td>3 / 3</td>
<td>2.27e-04</td>
</tr>
<tr>
<td>Signaling by FLT3 fusion proteins</td>
<td>3 / 20</td>
<td>0.002</td>
<td>0.019</td>
<td>0.578</td>
<td>17 / 18</td>
<td>0.001</td>
</tr>
<tr>
<td>Complex I biogenesis</td>
<td>5 / 55</td>
<td>0.005</td>
<td>0.02</td>
<td>0.578</td>
<td>6 / 13</td>
<td>9.83e-04</td>
</tr>
<tr>
<td>Purine catabolism</td>
<td>3 / 21</td>
<td>0.002</td>
<td>0.021</td>
<td>0.578</td>
<td>5 / 28</td>
<td>0.002</td>
</tr>
<tr>
<td>SUMOylation of DNA damage response and repair proteins</td>
<td>6 / 81</td>
<td>0.007</td>
<td>0.027</td>
<td>0.578</td>
<td>7 / 24</td>
<td>0.002</td>
</tr>
<tr>
<td>RHOGTPases activate KIN1</td>
<td>2 / 11</td>
<td>9.52e-04</td>
<td>0.038</td>
<td>0.578</td>
<td>2 / 2</td>
<td>1.51e-04</td>
</tr>
<tr>
<td>Apoptotic cleavage of cell adhesion proteins</td>
<td>2 / 11</td>
<td>9.52e-04</td>
<td>0.038</td>
<td>0.578</td>
<td>2 / 10</td>
<td>7.56e-04</td>
</tr>
<tr>
<td>FLT3 signaling in disease</td>
<td>3 / 30</td>
<td>0.003</td>
<td>0.032</td>
<td>0.578</td>
<td>24 / 52</td>
<td>0.004</td>
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<tr>
<td>Diseases of Telomere Maintenance</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>2 / 2</td>
<td>1.51e-04</td>
</tr>
<tr>
<td>Alternative Lengthening of Telomeres (ALT)</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>2 / 2</td>
<td>1.51e-04</td>
</tr>
<tr>
<td>Defective Inhibition of DNA Recombination at Telomere</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>2 / 2</td>
<td>1.51e-04</td>
</tr>
<tr>
<td>Defective Inhibition of DNA Recombination at Telomere Due to ATRX Mutations</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>1 / 1</td>
<td>7.56e-05</td>
</tr>
<tr>
<td>Defective Inhibition of DNA Recombination at Telomere Due to DAXX Mutations</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>1 / 1</td>
<td>7.56e-05</td>
</tr>
<tr>
<td>Defective LARGE causes MDC1GAS and MDC1GIs</td>
<td>1 / 2</td>
<td>1.75e-04</td>
<td>0.054</td>
<td>0.578</td>
<td>1 / 1</td>
<td>7.56e-05</td>
</tr>
<tr>
<td>Regulation of HSF1-mediated heat shock response</td>
<td>5 / 75</td>
<td>0.007</td>
<td>0.06</td>
<td>0.578</td>
<td>6 / 14</td>
<td>0.001</td>
</tr>
<tr>
<td>Regulation of necroptotic cell death</td>
<td>3 / 32</td>
<td>0.003</td>
<td>0.061</td>
<td>0.578</td>
<td>5 / 18</td>
<td>0.001</td>
</tr>
<tr>
<td>Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins</td>
<td>7 / 125</td>
<td>0.011</td>
<td>0.062</td>
<td>0.578</td>
<td>13 / 31</td>
<td>0.002</td>
</tr>
<tr>
<td>Respiratory electron transport</td>
<td>6 / 91</td>
<td>0.009</td>
<td>0.065</td>
<td>0.578</td>
<td>10 / 19</td>
<td>0.001</td>
</tr>
<tr>
<td>Transcriptional Regulation by E2Fs</td>
<td>3 / 34</td>
<td>0.003</td>
<td>0.07</td>
<td>0.578</td>
<td>11 / 33</td>
<td>0.002</td>
</tr>
<tr>
<td>PTK6 Down-Regulation</td>
<td>1 / 3</td>
<td>2.62e-94</td>
<td>0.08</td>
<td>0.578</td>
<td>3 / 3</td>
<td>2.27e-04</td>
</tr>
<tr>
<td>Signaling by TCF7L2 mutants</td>
<td>1 / 3</td>
<td>2.62e-94</td>
<td>0.08</td>
<td>0.578</td>
<td>1 / 1</td>
<td>7.56e-05</td>
</tr>
<tr>
<td>GLI protein bind promoters of Hes responsive genes to promote transcription</td>
<td>1 / 3</td>
<td>2.62e-94</td>
<td>0.08</td>
<td>0.578</td>
<td>3 / 4</td>
<td>3.02e-04</td>
</tr>
<tr>
<td>Electron transport from NADPH to Ferredoxin</td>
<td>1 / 3</td>
<td>2.62e-04</td>
<td>0.08</td>
<td>0.578</td>
<td>1 / 2</td>
<td>1.51e-04</td>
</tr>
<tr>
<td>RIPK1-mediated regulated necrosis</td>
<td>3 / 36</td>
<td>0.003</td>
<td>0.08</td>
<td>0.578</td>
<td>7 / 54</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Table 3.5 – Clinical implications of well-known Cul3 substrates, substrate adaptors, and second messengers:
Five well-characterized proteins that have been previously linked to Cul3 were identified in our MS analysis. First column shows the name and gene of the proteins, second column shows their fold changes, third column shows how the molecular connection between the proteins and Cul3, fourth column shows the clinical implications of the protein, and fifth column shows the most recent reference describing the protein roles.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold change</th>
<th>Cul3 roles</th>
<th>Clinical Implications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras Homolog Family Member A (RhoA)</td>
<td>2.0</td>
<td>Substrate of Cul3-Bacurd complex</td>
<td>Familial hyperkalemia and hypertension (FHHt)</td>
<td>Chen et al., 2009</td>
</tr>
<tr>
<td>Ras Homolog Family Member B (RhoB)</td>
<td>1.5</td>
<td>Substrate of Cul3/Kctd10 complex</td>
<td>Apoptosis in neoplastically transformed cells after DNA damage</td>
<td>Murakami et al., 2019</td>
</tr>
<tr>
<td>With no lysine 1 (WNK1)</td>
<td>1.4</td>
<td>Substrate of Cul3-Klhl3 complex</td>
<td>Familial hyperkalemia and hypertension (FHHt)</td>
<td>Cornellius et al., 2018</td>
</tr>
<tr>
<td>Rac Family Small GTPase 1 (Rac1)</td>
<td>1.4</td>
<td>Ubiquitinated RhoB inhibits Rac1 activation</td>
<td>Apoptosis in neoplastically transformed cells after DNA damage</td>
<td>Murakami et al., 2019</td>
</tr>
<tr>
<td>Ankyrin repeat and FYVE domain-containing protein 1 (ANKFY1)</td>
<td>10</td>
<td>Substrate adaptor of Cul3 in the Cul3-ANKFY1-integrin β1 complex</td>
<td>Regulation of endosomal membrane traffic and angiogenesis</td>
<td>Maekawa et al., 2017</td>
</tr>
</tbody>
</table>
Table 3.6 – Clinical implications of potential Cul3 substrates involved in FHH: Four kidney proteins that have not been characterized as Cul3 targets were identified in our MS analysis. First column shows the name and gene of the proteins, second column shows their fold changes, third column shows how the molecular connection between the proteins and Cul3, fourth column shows the clinical implications of the protein, and fifth column shows the most recent reference describing the protein roles.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold change</th>
<th>Expression in the kidney</th>
<th>Clinical Implications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin</td>
<td>2.6</td>
<td>Distal convoluted tubule (DCT), intercalated cells and principal cells of cortical and medullary collecting ducts, and in ureter</td>
<td>Facilitates removal of potentially inflammatory actins released from injured cells</td>
<td>Lueck et al., 1998</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 1 (ERK2)</td>
<td>2.5</td>
<td>Distal convoluted tubule (DCT)</td>
<td>It mediates cell response to injuries in the kidney and downregulates NCCs</td>
<td>Capolongo et al., 2019</td>
</tr>
<tr>
<td>Syntaxin 4A (STX4A)</td>
<td>2.0</td>
<td>It is expressed in renal collecting duct principal cells and is localized to the apical plasma membrane</td>
<td>It targets aquaporin-2-containing vesicles to the apical plasma membrane</td>
<td>Mandon et al., 1996</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 3 (ERK1)</td>
<td>1.75</td>
<td>Distal convoluted tubule (DCT)</td>
<td>It mediates cell response to injuries in the kidney and downregulates NCCs</td>
<td>Buscà et al., 2016</td>
</tr>
</tbody>
</table>
Table 3.7 – Clinical implications of potential Cul3 substrates not involved in FHHt: Two tumor suppressors that have not been characterized as Cul3 targets were identified in our MS analysis. First column shows the name and gene of the proteins, second column shows their cellular roles, third column shows the clinical implications of the protein, and fourth column shows the most recent reference describing the protein roles.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular Role</th>
<th>Clinical Implications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2 and CDKN1A-interacting protein (BCCIP)</td>
<td>Important cofactor for BRCA2 in tumor suppression, and a modulator of CDK2 kinase activity via p21</td>
<td>Maintenance of genome stability and its downregulation is correlated with the aggressiveness of brain tumors</td>
<td>Liu et al., 2009</td>
</tr>
<tr>
<td>Rb1-inducible coiled-coil protein 1 (RB1CC1)</td>
<td>Potent regulator of the RB1 pathway through induction of RB1 (Retinoblastoma 1) expression</td>
<td>RB1CC1 regulates cell growth, cell proliferation, apoptosis, autophagy, and cell migration, and its deregulation is associated with cell growth and progression of various cancers</td>
<td>Kontani et al., 2003</td>
</tr>
</tbody>
</table>
Table 3.8 – Summary of proteins that contain conserved domains of interest from MS-based proteomic analysis. First column shows the different domains observed among the altered proteins, second column shows the protein names, and third column shows their UniProt© accession number (The UniProt Consortium, 2021)

<table>
<thead>
<tr>
<th>Domains</th>
<th>Protein name</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD40</td>
<td>WD repeat protein BING4</td>
<td>O15213</td>
</tr>
<tr>
<td></td>
<td>WD repeat protein 4</td>
<td>P57081</td>
</tr>
<tr>
<td></td>
<td>WD repeat protein 1</td>
<td>O75083</td>
</tr>
<tr>
<td></td>
<td>WD repeat endosomal protein</td>
<td>Q8TAF3</td>
</tr>
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<td></td>
<td>WD repeat domain 82</td>
<td>Q6UXN9</td>
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<td>WD repeat domain 79</td>
<td>E9MRK3</td>
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<td>WD repeat domain 76</td>
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<td></td>
<td>WD repeat domain 75</td>
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<td>WD repeat domain 74</td>
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<td>WD repeat domain 50</td>
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<td></td>
<td>WD repeat domain 12</td>
<td>Q9G2L7</td>
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<tr>
<td></td>
<td>Glutamate rich WD repeat containing 1</td>
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<td>Coronin 1B</td>
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<td>Coronin 2A</td>
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<td></td>
<td>Coronin 1C</td>
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<td></td>
<td>Coronin 1A</td>
<td>P31146</td>
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<td>WD40 and HMG-box</td>
<td>WD repeat and HMG box DNA binding protein 1</td>
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<td>WD40 and FYVE</td>
<td>WD repeat and FYVE domain containing 1</td>
<td>Q81WB7</td>
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<tr>
<td>WD40 and PI</td>
<td>Peptidylprolyl isomerase domain and WD repeat containing 1</td>
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<td>UPS</td>
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<td>Ubiquitin specific protease 6</td>
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<td>Ubiquitin specific protease 7</td>
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<td>Ubiquitin specific protease 15</td>
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<td>Ubiquitin specific protease 19</td>
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<tr>
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Figure 3.6 – Proposed mechanisms of control for the proteins with low spectral counts: In Cul3 KO cells, intermediary regulators are likely to be ubiquitinated by Cul3 and targeted to degradation by the 26S proteasome. However, in Cul3 KO cells, they can no longer be ubiquitinated, which leads to a rise in their cellular levels and, consequently, a reduction in the levels of their target proteins.
Figure 3.7 – Validation of the proteomics analysis: HEK 293 WT and Cul3 KO cells were co-transfected with HA-UbE2D1 or HA-LRR1. Immunoblotting was used to probe for endogenous Cul3 (first rows), HA-UbE2D1 and HA-LRR1 (second rows), control Actin (third rows), and quantification of the western blot bands (fourth rows). (A) Protein with high spectral count: expression levels of UbE2D1 in KO and WT cells. (B) Protein with low spectral count: expression levels of LRR1 in KO and WT cells.
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CHAPTER 4

Discussion and Future Directions
DISCUSSION

This dissertation focuses on the structure and function of a ubiquitin E3 ligase. Chapter 2 describes a series of experiments focusing on a hypothesis that the protein that helps the complex identify substrates may also participate in the selection of the E2 enzyme. Chapter 3 describes a proteomics approach to testing the hypothesis that Cul3 regulates yet unidentified additional substrates in kidney cells. Below, I will discuss different regulatory mechanisms involved in the function of the Cul3 complex, the significance of our findings, and the relationship between the two approaches taken to characterize the Cul3-based ubiquitin ligase. Lastly, we will also propose future experiments to continue the thorough characterization of the mammalian Cul3 complex and support its relevance at the cellular and organismal levels.

THE POTENTIAL ROLE OF CUL3 ADAPTORS IN E2 SELECTION

The chemistry behind the conjugation of ubiquitin to its substrate has been rigorously studied, but the mechanism of E2 recruitment and polyubiquitin chain formation has not yet been elucidated (Hochstrasser, 2006). The majority of the information regarding these processes comes from finding out that the binding site of the ubiquitin-activating enzyme and the ubiquitin ligase overlap on the surface of the ubiquitin-conjugating enzyme. In addition, the order in which the ubiquitin molecule can be transferred in the ubiquitination pathway is limited because the
ubiquitin-conjugating enzyme cannot bind the ubiquitin-activating enzyme and the ubiquitin ligase simultaneously. (Eletr et al., 2005; Kleiger et al., 2009).

Several hypotheses have been raised to explain the mechanisms of E2 recruitment and substrate ubiquitination (Hochstrasser, 2006). The most popular one is the traditional sequential model that proposes that the ubiquitin molecules are transferred from an E1 enzyme to an E2 enzyme that binds to Cul3. Then, together, they transfer the ubiquitin to the substrate. (Figure 4.1A). Several studies have also indicated that the recruitment of the E2 is entirely random and occurs independently of the substrate that is being ubiquitinated. In addition, models have suggested that the recruitment of E2s for the ubiquitin-conjugation process occurs independently of the substrate that is being ubiquitinated and involves the C-terminus of Cul3 interacting with the ubiquitin-conjugating enzyme (E2) and the N-terminus interacting with and recruiting the substrate via a BTB domain-containing protein (Figure 4.1A).

In the study presented here, I have made three important observations about this subject. First, I determined that Cul1 and Cul3 have differential binding preferences for E2s. Second, I determined that the N-terminus region of Cul3 contained the E2 binding specificity of the wild-type Cul3. Third, a BTB adaptor protein is required for this interaction to occur, which implies that the BTB domain-containing proteins are likely to be involved in the E2 recruitment and certain E2 enzymes can interact with both termini of Cul3 (Figure 4.1B).
Undoubtedly, these results provide strong evidence supporting the hypothesis that BTB domain-containing proteins participate in the E2 selection process. However, many questions remain unanswered regarding this novel mechanistic insight, including:

(1) Does the Cul3 complex always utilize this mechanism to choose E2s?
(2) What part of the BTB domain-containing protein is involved in this novel E2-BTB domain-containing protein interaction?
(3) What part of the E2 protein is involved in binding the BTB domain-containing protein?
(4) What, if any, role does the previously characterized Rbx1 recruitment play in E2 selection?

These questions center around three main protein families: BTB domain-containing proteins, ubiquitin-conjugating enzymes (E2s), and Cullins. As discussed in the introduction, we know a lot about the functional domains of each of these families. In the following sections, I will discuss their classes, structures, and functional domains, consider the implications of each question, and suggest strategies on how to address them.

I. **BTB domain-containing proteins**

The BTB domain-containing protein gene family is characterized by the presence of one or more BTB domains in each family member and is found widely
in eukaryotes (Zollman et al., 1994). The human genome encodes 188 BTB proteins that can be divided into subfamilies according to the presence or absence of additional domains, including BTB only proteins, BTB-ZF proteins, BTB-Kelch proteins, BTB-BACK proteins, BTB-BACK-Kelch proteins, MATH-BTB proteins, BTB-ANK proteins, BTB-BACK-PHR proteins, and Rho-BTB proteins (Figure 4.2; Stogios et al., 2005; Li et al., 1999). As mentioned in chapter 1, they function as substrate adaptors during protein ubiquitination by the Cul3 complex. In these proteins, the BTB domain is proposed to interact with Cul3, whereas the second domain, such as the ZF, Kelch, ANK, or MATH domain, is presumed to act as the substrate-recognition module (Boeckmann et al., 2004).

Despite the vast diversity, there are not many known BTB domain-containing proteins capable of interacting with ubiquitin-conjugating enzymes. In chapter 2, we demonstrated that RhoBTB3 could interact with UbE2E1, but besides this result, only a BTB-BACK-Kelch family member has been shown to bind to an E2. The structure and functional domains of these two BTBs vary drastically, suggesting that they are likely using distinct domains to interact with the E2s. Regarding the BTB-BACK-Kelch protein, its recently identified BACK domain has not yet a defined function (Stogios & Prive, 2004). We hypothesize that it may be a vital component for the protein-protein interaction with the E2. To test this, we can delete it from the BTB-BACK-Kelch protein and examine the binding to the E2. Regarding the domain that RhoBTB3 uses to interact with
UbE2E1, we have available a unique set of mutants that can be used in further binding tests.

II. Ubiquitin-conjugating enzymes (E2s)

As described in Chapter 1, much is known about their structure and functional domains. In the human genome, there are 35 E2s exclusively dedicated to ubiquitin conjugation. They are grouped according to the presence of appendages either N- or C-terminus to the catalytic domain (Classes II and III, respectively), at neither (Class I), or at both ends (Class IV). They all share a ~150-amino acid conserved catalytic core domain, known as the ubiquitin conjugation (UBC) domain, which is the minimal sufficient unit for the E2 enzymatic activity. We hypothesize that their appendages may be involved in the binding interaction with BTB domain-containing proteins. To test this, we can delete them from UbE2E1 and test for changes in binding to RhoBTB3. We can also use the fact that we have several related E2s, some of which bind and many that do not to do an alignment, to attempt to identify unique sequences found in common with binders.

III. Cullins

As described in chapter 2, we developed cullin chimeras in which we fused the N- or C-terminus of Cul1 to the N- or C-terminus of Cul3 (Figure 2.1). We can take advantage of this unique set of tools to characterize the role of Cullins domains in the interaction with E2s and, possibly, expand our knowledge on this novel
selection mechanism. A crucial question that needs to be addressed is what, if any, role does the previously characterized Rbx1 recruitment play in E2 selection? We have the tools to examine this question, a mutant that does not bind Rbx1 was created and characterized in our laboratory and would be an invaluable tool for such an analysis (Wimuttisuk & Singer, 2007). Another critical question that needs to be answered is what region of the N-terminus Cul3 is responsible for the interaction with the E2s? Besides the mutant that cannot bind to Rbx1, we have at our disposal a variety of other mutants with specific deletions that would be very helpful in pinpointing the binding region.

**POTENTIAL PATHWAYS OF CUL3 INTERACTIONS AND NOVEL COMPONENTS OF THE CUL3 COMPLEX**

Since its discovery, Cul3 has emerged as a critical player in recognizing and recruiting numerous important substrates for ubiquitination. The heterogeneity of substrates, substrates adaptors, intracellular and intercellular processes in which Cul3 is involved demonstrates this E3 ligase’s relevance at both the cellular and organismal level. Although progress has been awe-inspiring over the past decades, there is still much that remains to be discovered to understand this intriguing class of Cullin-RING ubiquitin ligases fully. In the following sections, I examine the cellular effects of Cul3 dysregulation as well as how this can lead to the development of several disorders. In addition, I discuss the role of Cul3 in cell signaling and propose a novel model for the BCR complex.
I. Correlations between Cul3 substrates and diseases

The characterization of the Cul3 complex provides a better understanding of the ubiquitination process and may also offer preventative strategies and treatments for diseases caused by any disruptions in the Cul3 gene and misregulation of its substrates. Our first chapter demonstrated the importance of the Cul3-based ubiquitin ligase by providing a short description of Cul3 target proteins and describing some of the Cul3 roles in different diseases. For example, accumulation of cyclin E, a well-known Cul3 substrate, could lead to breast, ovarian, and colon cancers (Buckley et al., 1993; Guardavaccaro and Pagano, 2004; Hunt and Keyomarsi, 2005; Rosen et al., 2006). In addition, misregulation of the transcription factor Nrf2, which is a multi-organ protector against oxidative stress response, has been linked to the onset of several diseases, including carcinomas, Parkinson's disease, and lupus autoimmune disease (Yoh et al., 2001; Yu and Kensler, 2005; Burton et al., 2006; Hayes and McMahon, 2006).

Overexpression of WNK1 and WNK4, which regulate the activity of the major sodium and potassium transporters in the distal nephron (NCC), is the leading cause of hypertension and hyperkalemia symptoms observed in patients with Familial hyperkalemic hypertension (FHHt; McCormick et al., 2014; Cornelius et al., 2018). Furthermore, failure to ubiquitinate Cul3 substrates involved in X-chromosome inactivation may lead to male-lethal X-linked traits, such as focal-dermal hypoplasia and oral-facial-digital syndrome type 1 (Franco and Ballabio,
These substrates include H2AFY and possibly a novel Cul3-binding protein named RBM10. Additionally, two potential Cul3 substrates identified in our MS-based proteomic analysis, known as ERK1 and ERK2, are likely to play crucial roles in their cellular pathways as well as in the developmental process of FHHt. Therefore, the characterization of the Cul3-based ubiquitin ligase will contribute to a greater understanding of the underlying cause and potential treatments of diseases stemming from the misregulation of the Cul3-based ubiquitin ligase complex.

II. Role of Cul3 in cell signaling

The E3 ubiquitin-protein ligase RSP5 catalyzes the mono-, di-, and multi-ubiquitination of different substrates that serve as signals for various cellular pathways, including the 26S proteasome, endocytosis, and DNA repair (Galan and Haguenauer-Tsapis, 1997; Huibregtse et al., 1997; Dunn and Hicke, 2001; Hicke, 2001). Contrastingly, the most prominent role of Cul3-based E3 ligase is to ubiquitinate and target substrates for degradation by the 26S proteasome. However, a growing body of research has shown that Cul3 can catalyze the assembling of different ubiquitin linkages that serve as unique signaling molecules not just for the 26S proteasome pathway but also for non-proteolytic signaling processes (Jerabkova & Sumara, 2019; Maerki et al., 2009; Sumara & Peter, 2007; Bade et al., 2014). Furthermore, Cul3 has been shown to catalyze the formation
of polyubiquitin chains as well as the catalyzation of monoubiquitin attachments (Hernandez-Munoz et al., 2007; Sumara et al., 2007).

Multiple well-known Cul3 substrates have been identified in our analysis (Chapter 3). Most of them are known to be polyubiquitinated by Cul3 in the lysine 48 and targeted for degradation by the 26S proteasome. However, a subset of substrates known to be either monoubiquitinated or polyubiquitinated in a different lysine was also identified, revealing that the absence of Cul3 has more cellular implications than just the overexpression of unwanted substrates. Besides the proteasome pathway, DNA synthesis and repair, development, vesicle transport, and cytoskeletal remodeling were some of the signaling processes disrupted in our Cul3 KO cells (Table 4.1). To further understand the ubiquitination via Cul3, the process of lysine selection, and the assembly of ubiquitin linkages, we will explore possible mechanisms for substrate recognition and the signaling events required to activate the Cul3-based ubiquitin ligase complex.

III. WD40/LRR domain-containing proteins function as substrate adaptors for Cul3

Identifying common conserved motifs in our MS-based proteomic analysis revealed a subset of proteins that contained either WD40 or LRR domains (Chapter 3). At first, we were inclined to categorize these proteins as potential substrates for the Cul3-based ubiquitin ligase complex. However, a study conducted by our group in which we found a new set of Cul3-bound proteins that
contained either the LRR or WD40 domain made us reconsider our hypothesis. In addition, they also demonstrated that these LRR domain-containing proteins could bind both Cul3 and BTB domain-containing proteins, and this dual binding role for the LRRs caused the BTB-domain protein to become a substrate instead of an adaptor (Wimuttisuk et al., 2014). Furthermore, these domains are commonly found as part of the F-box and DWD proteins, which serve as substrate adaptors for the SCF (Skp1-Cul1-F-box) ubiquitin ligase complex and the DDB1-Cul4A ubiquitin ligase complex, respectively (Zheng et al., 2002; Angers et al., 2006a; He et al., 2006), and a current model of the BCR ubiquitin ligase proposes that the BTB domain-containing protein recruits its substrate using a second protein-protein interacting domain, such as a Kelch or a MATH domain. Thus, we proposed that Cul3 may acquire an additional substrate specificity module by binding to WD40/LRR domain-containing proteins, and they then serve as F-box-like substrate adaptors for the complex.

IV. Potential substrates involved in the severe phenotype of FHHt

Familial Hyperkalemic Hypertension (FHHt)

As discussed in chapter 3, FHHt is an inherited disorder manifested by hyperkalemia and hypertension caused by the hyperactivation of the NCCs that are expressed exclusively in the DCTs (Mayan et al., 2002; Isobe et al., 2012). For the past two decades, considerable efforts have been taken to identify the proteins involved in FHHt, which revealed that mutations in CUL3, its substrate adaptor
KLHL3 and its substrates WNK1 and WNK4 can also lead to FHHt, with CUL3 knockouts causing the most severe form of FHHt. The phenotypic severity of Cul3 knockouts compared to the phenotypes observed in the other three proteins suggests that other contributing factors besides Klhl3-Wnk1/Wnk4 pathway that are leading to this phenotypic difference.

Through an unbiased proteomic screen, I identified a subset of upregulated proteins in the KO cells that have the potential to be Cul3 substrates and possibly be involved in FHHt, including ERK1, ERK2, STX4A, and GSN. After a careful analysis, my results supported the hypothesis that ERK1 and ERK2 have the potential to be Cul3 substrates and, in collaboration with WNK1 and WNK4, they likely regulate the activity of the NCCs.

ERK1 and ERK2 are responsible for mediating cellular responses to injuries and stress in the kidneys as well as the regulation of the NCCs. As mentioned in chapter 3, upregulation of NCCs is the main cause of the increased blood pressure and elevation of potassium levels observed in FHHt patients are triggered by disruption of Cul3 and, as a result, enhanced activity of its substrates WNK1 and WNK4. The fact that both WNKs and ERKs are kinases and control the activity of NCCs in the DCT supports the hypothesis that the severe phenotype observed in Cul3 knockouts is caused by simultaneous enhanced activity of multiple substrates, and the ERKs are possibly part of this regulatory pathway.
V. Mechanisms of action and potential pathological consequences of abnormal ERK signaling in FHHt

ERK1 and ERK2 are mitogen-activated protein kinases (MAPKs), which are proteins responsible for initiating the activation of signaling pathways that act as intracellular communication lines and contribute to structural and functional cellular responses. They are mainly activated by stress stimuli, and are, therefore, sometimes categorized as stress kinases (Kurtzeborn et al., 2019).

My hypothesis proposes that ERK1 and ERK2 are likely to be ubiquitinated by Cul3 and targeted to degradation by the 26S proteasome. However, in Cul3 KO cells, they can no longer be ubiquitinated, leading to a rise in their cellular levels and, consequently, over-stimulating the kidney pathways in which they are involved. As mentioned above, ERK1 and ERK2 mediate the activation of NCCs, thus the over-expression of these two MAPKs likely enhances NCC activity, which in turn contributes to the kidney tissue injury observed in severe cases of FHHt (Figure 4.3).

To test my hypothesis that ERK1 and 2 are Cul3 substrates, I will have to perform a series of biochemical tests, including (1) Examine if the levels of these two MAPKs are higher in the Cul3 KO cells. This can be achieved by transfecting them into WT and Cul3 KO cells and comparing their expression levels. (2) Test their binding capability to the Cul3 complex through immunoprecipitation assays. Then, I can take advantage of the Cul3 ΔBTB and compare the binding to the wild-
type Cul3. (3) Through ubiquitination assays, determine if the ubiquitination levels of ERK1 and ERK2 change in the presence and absence of Cul3.

**MECHANISMS OF SUBSTRATE RECOGNITION BY CUL3**

Cul3 selects protein substrates for the ubiquitination pathway by recognizing a specific signal that is common only among its target proteins. Several studies have mapped the BTB-binding regions on various Cul3 substrates. However, such analyses have yet to reveal a consensus signal that initiates the substrate selection process for Cul3. Although most substrates are targeted for ubiquitination by a single cullin E3 ligase, emerging evidence demonstrates that a few substrates can be ubiquitinated by several cullins. For instance, Maeda and colleagues' *in vitro* ubiquitination assay revealed that both the Cul1 and Cul3 could catalyze the polyubiquitination of cyclin D (Maeda et al., 2001). Even though it has been well established that Cul3 catalyzes the ubiquitination and the subsequent degradation of cyclin E in mice (Singer et al., 1999), a couple of studies have shown that cyclin E is also a substrate of the SCF$^{\text{fbw7}}$ complex (Koepp et al., 2001; Strohmaier et al., 2001). Lastly, both Cul1 and Cul3 are responsible for the ubiquitination of the Ci protein, which is a regulator of the hedgehog signaling pathway involved in the development of the *Drosophila* eye (McEvoy et al., 2007; Ou et al., 2002). In the following sections, I explore potential mechanisms of substrate recognition and examine different cellular signaling events that may play a role in the activation of the Cul3 complex and the recognition of its substrates.
I. Temporal regulation

The identification of substrates by cullins has focused primarily on the interactions between their substrate adaptors and target proteins. An alternative hypothesis for this model is that the ubiquitination pathway is under a temporal regulation, and each cullin is only activated under specific cellular conditions. The ubiquitination of the p53 protein is an interesting example of such regulation, in which a single substrate is targeted for degradation by at least five different ubiquitin ligases, including Mdm2, Cul7, ARF-BP1, COP1, and Pirh2 (Brooks and Gu, 2006). Even though most of these ubiquitin ligases become functional by a negative feedback loop that regulates the expression levels of p53, a few of them are activated under specific cellular conditions. For example, Pirh2 targets p53 for degradation in response to cellular damage (Duan et al., 2006), while Cul7 ubiquitinates p53 to promote cell growth (Andrews et al., 2006). Similarly, substrate ubiquitination by Cul3 might be triggered by different cellular responses that have not yet been characterized.

II. Spatial regulation

The proximity of the target substrate with its ubiquitin ligase may lead to substrate recognition and consequent activation of the ubiquitin ligase, which would indicate that spatial regulation may play a role in the induction of the ubiquitin ligase activity (Pines and Lindon, 2005). Therefore, it is possible that different cullin ligases are not competing for the same substrate but rather coordinating their
functions in separate cellular compartments or tissues based on their localization. Ou and colleagues tested this hypothesis in a study about the development of the Drosophila eye. They showed that both Cul1 and Cul3 are responsible for the degradation of Ci during the third instar larva period (Ou et al., 2002). Although Cul1 and Cul3 separately ubiquitinate Ci in the anterior and the posterior regions to the morphogenetic furrow, respectively, the expression of these cullins is not limited to the region in which they are functionally active. For instance, Cul1 was detected in the posterior of the morphogenetic furrow where Cul3 alone targets the ubiquitination of Ci, indicating that the presence of Cul1 in proximity to a potential substrate does not activate its function as a ubiquitin ligase. Therefore, spatial regulation does not necessarily influence either substrate selection or the activation of cullin ubiquitin ligases. However, the possibility that substrate selection is affected by the localization of cullin substrate adaptors or the ubiquitin-conjugating enzymes (E2s) remains unknown.

III. Binding based on specific types of protein-protein interactions:

A specific ubiquitin ligase might recognize different groups of substrates differentiated by either post-translational modifications or complex formation with other proteins. For instance, it has been determined that the SCF\textsuperscript{fbw7} complex specifically binds cyclin E that has been phosphorylated at the Thr\textsuperscript{380} residue (Koepp et al., 2001). Still, another study suggested that Cul3 is more likely to recognize the non-phosphorylated form of cyclin E (Singer et al., 1999).
Additionally, during the development of the Drosophila eye, phosphorylation by protein kinase A (PKA) is essential for the Cul1-mediated degradation of the Ci protein, as shown by the reduction of Ci expression in the presence of a constitutively active PKA. However, the ability of Cul3 to ubiquitinate Ci substrates is entirely independent of PKA activity, which further supports the hypothesis that Cul1 and Cul3 mediate the ubiquitination of different subtypes of the same target protein. Even though phosphorylation provides a recognition signal for Cul1 substrates, a substrate recognition factor for the Cul3-based ubiquitin ligase has not yet been confirmed. By analyzing the interaction between Cul3 and the proteins listed in Table 3.6, which is about the common domains found in the altered proteins of our MS-based proteomic analysis, we may acquire valuable information regarding the molecular basis of substrate recognition by the Cul3-based E3 ligase complex.

IV. Coordinated activity of different cullins and mechanisms of compensation

It is also crucial to consider that multiple ubiquitin ligases targeting the same substrate may confer a coping mechanism for cells and organisms. By allowing the substitution of one ubiquitin ligase enzyme for another, an organism may compensate for other mutations in the ubiquitination machinery. For instance, the cell cycle regulator cyclin E is a well-characterized Cul3 substrate (Davidge et al.,
2019), but studies have shown that both Cul1 and Cul3 are involved in its degradation (Clurman et al. 1996; Singer et al. 1999; Petroski and Deshaies 2005).

CONCLUSIONS

Cul3-based E3 ligase is responsible for regulating a variety of cellular pathways, many of which are known to have profound effects on the proper function of multicellular organisms. Thus, we began this journey trying to elucidate the physiological context in which this E3 ligase operates in mammalian cells, in which we expected to identify relevant E2 partners, understand the role of BTB domain-containing proteins in the recruitment of E2s, identify potential Cul3 substrates, and get a better understanding of their biological functions.

First, I have discovered that BTB adapter proteins are probably involved in the E2 recruitment. Secondly, I proposed that the WD40/LRR domain-containing proteins are likely to be substrate adaptors for the Cul3 complex, not substrates. Third, the severe phenotype of Cul3 knockouts observed in FHHt, is likely to be caused by the over-expression of multiple factors, including the two potential Cul3 substrates, ERK1 and ERK2, identified in my proteomics screen. Lastly, identifying potential members of the Cul3 complex using the MS-based proteomic approach has established a correlation between Cul3 and the over-activation of the NCC pathway observed in FHHt. I hope that the knowledge gained from this dissertation will be applied to achieve the ultimate goal that is to fully characterize the
ubiquitination mechanism of the Cul3-based E3 ligase at the cellular and organismal levels.
Figure 4.1 – Models of E2 recruitment and substrate ubiquitination: (A) Current suggested model of E2 recruitment and assembly with the Cul3 complex. (B) Proposed model of E2 recruitment and assembly with Cul3.
Figure 4.2 – Structures and protein architectures of four BTB domain-containing proteins that function as Cul3 substrates adaptors: A) KEAP1 is composed by NTR (N-terminal region), BTB, intervening region (IVR), DC domain harboring six Kelch-repeat domain and C-terminal region (CTR). BTB domain and N-terminal portion of IVR are important for the association with CUL3. DC domain mediates interaction with Neh2 domain of NRF2. B) SPOP contains a MATH domain for substrate binding, a BTB domain that binds to CUL3, a C-terminal domain (CTD) that contains a dimerization interface, and a nuclear localization sequence (NLS). C) KLHL3 is composed by a BTB domain, a BACK domain, and six Kelch repeats. D) LZTR1 is an unusual BTB-Kelch protein with six Kelch repeats present in the N-terminus followed by two BTB-BACK domains.
Receptor tyrosine kinase
Ligand
Extracellular
Cytoplasm

RAS
MAP3K
MAP2K
MAPK

RAF
MEK1/2
ERK1/2

Cul3 knockout

No ubiquitination by Cul3
No degradation by the 26S proteasome

Overexpression of ERK1/2

NCC overactivation

NCC

Na+ retention
Hypertension

Cul3
Rbx1
BTB
E2
Figure 4.3 - Schematic summary of the ERK1 and ERK2 pathway: The ligand (pink triangle) binds to the dimeric transmembrane tyrosine kinase receptor (blue/salmon blocks) and activates a spectrum of downstream intracellular cascade. The MAPK pathway is highlighted in the light gray box and mediates extracellular information to the cell interior. The last step of ERK cascade activation results in phosphorylation of ERK1/2 leading to activation of the NCCs. In Cul3 knockout cells, ERKs cannot be ubiquitinated by Cul3 and degraded by the 26S proteasome. Consequently, ERK1/2 levels rise and overactivation of NCC happens.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Type of Ubiquitination</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurora kinase B</td>
<td>Mono</td>
<td>Regulation of mitotic localization and chromosome segregation</td>
</tr>
<tr>
<td>H2AFY</td>
<td>Mono</td>
<td>Stable X chromosome inactivation</td>
</tr>
<tr>
<td>Sec31</td>
<td>Poly (K33)</td>
<td>Assembly of large COPII coated vesicles and collagen secretion</td>
</tr>
<tr>
<td>MCM3</td>
<td>Poly (K48)</td>
<td>Loading and regulation of DNA replication</td>
</tr>
</tbody>
</table>


Hicke, L. (2001). Protein regulation by monoubiquitin. *Nature Reviews Molecular Cell Biology, 2*(3), 195–201. [https://doi.org/10.1038/35056583](https://doi.org/10.1038/35056583)


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https://doi.org/10.1210/jcem.87.7.8449

https://doi.org/10.1172/JCI76126


