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Novel Roles for the E3 Ubiquitin Ligase Parkin in the Nucleus

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Sarah E. Shires

Committee in charge:

Professor Åsa Gustafsson, Chair
Professor Alexandra Newton
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2019

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Chair

University of California San Diego

2019

DEDICATION

One of my favorite parts of reading Harry Potter is the excitement I feel each time Harry finds a new mentor to look up to and learn from, and the sadness I feel when they are taken away.

Mentors have made a huge difference in the story of my life, as well.

Therefore,

I dedicate this dissertation to all of my mentors.

EPIGRAPH

When you learn,
teach.
When you get,
give.

~ Maya Angelou

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LIST OF ABBREVIATIONS

2D	2 dimensional
ACADM	Acyl-CoA dehydrogenase, medium chain
ACADVL	Acyl-CoA dehydrogenase, very long chain
AMPK	Adenosine monophosphate(AMP)-activated protein kinase
ANF	Atrial natriuretic factor
ANOVA	Analysis of variance
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BNIP3L/NIX	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 Like
BPM	Beats per minute
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CD36	Cluster of Differentiation 36
cDNA	Complementary DNA
ChEA	Chromatin immunoprecipitation enrichment analyses
COX I	Mitochondrial complex I
COX II	Mitochondrial complex II
COX III	Mitochondrial complex III
COX IV	Mitochondrial complex IV
DEG	Differentially expressed gene

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Drp1	Dynamin related protein 1
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ERR α	Estrogen related receptor alpha
ERRE	Estrogen related receptor response element
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FDR	False discovery rate
Fis1	Fission protein 1
FS	Fractional shortening
FUNDC1	FUN14 domain containing 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal receptor growth factor 2
HIF	Hypoxia inducible factor
HK	Hexokinase

IBR	In-between-RING
I/R	Ischemia/reperfusion
IP	Immunoprecipitation
IVSd	Interventricular septal thickness at diastole
IVSs	Interventricular septal thickness at systole
KEGG	Kyoto encyclopedia of genes and genomes
LC3	Microtubule-associated protein 1 light chain 3
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LV	Left ventricle
LVEDD	Left ventricular end diastolic dimension
LVESD	Left ventricular end systolic dimension
LVPWd	Left ventricular posterior wall thickness at diastole
LVPWs	Left ventricular posterior wall thickness at systole
M199	Medium 199
MAPK	Mitogen-activated protein kinase
MDS	Multi-dimensional scaling
MEF	Mouse embryonic fibroblast
Mfn1/2	Mitofusin 1/2
MI	Myocardial infarction
Miro	Mitochondrial rho GTPase 1
MPP	Mitochondrial processing proteinase
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Mass spectrometry

mtDNA	Mitochondrial DNA
NBR1	Neighbor of BRCA gene 1
O/A	Oligomycin/Antimycin A
OCR	Oxygen consumption rate
P62/Sqstm1	Sequestosome 1
PARIS	Parkin-interacting substrate
Parkin ^{-/-} / Parkin KO	Parkin knockout
Parkin TG	Parkin transgenic
PARL	Presenilin associated rhomboid like
PD	Parkinson's Disease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC1 α/β	Peroxisome proliferator-activated receptor gamma coactivator 1- alpha/ beta
pH	Potential of hydrogen
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
PPARA/G	Peroxisome proliferator activated receptor alpha/gamma
PWM	Position weighted matrices
qPCR	Quantitative polymerase chain reaction
RBR	Ring-between-RING
RING	Really interesting new gene
RIPK3	Receptor-interacting serine/threonine-protein kinase 3

RNA-seq	Ribonucleic acid-sequencing
ROS	Reactive oxygen species
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum calcium ATP-ase
SIRT1	Sirtuin 1
TAC	Transverse aortic constriction
TCA	Tricarboxylic acid cycle
TIM23	Translocase of inner mitochondrial membrane 23
TFEC	Transcription factor EC
TL	Tibia length
TOM20	Translocase of the outer mitochondrial membrane 20
TRANSFAC	Transcription factor database
TUBE	Tandem ubiquitin binding entity
Ubl	Ubiquitin-like
UPS	Ubiquitin proteasome system
VPS	vacuolar proteins sorting
WT	Wild type
YFP	Yellow fluorescent protein

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ABSTRACTS

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The Dark Side of Parkin: The Non-mitophagy, Toxic Effects of Overexpressing Parkin. **Sarah E. Shires***, Mark A. Lampert, Dieter A. Kubli, Eileen R. Gonzalez, Nicole H. Purcell, and Åsa B. Gustafsson. BCVS AHA Scientific Sessions, Phoenix July 2016

Parkin Contributes to the Development of Cardiac Hypertrophy in Response to Cardiac Pressure Overload. **Sarah E. Shires***, Dieter A. Kubli, Eileen R. Gonzalez, Nicole H. Purcell, and Åsa B. Gustafsson. BCVS AHA Scientific Sessions, New Orleans July 2015

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ABSTRACT OF THE DISSERTATION

Novel Roles for the E3 Ubiquitin Ligase Parkin in the Nucleus

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2019

Professor Åsa Gustafsson, Chair

Parkin is an E3 ubiquitin ligase that facilitates clearance of damaged mitochondria by ubiquitinating proteins on the outer mitochondrial membrane. Although Parkin is mainly studied for its role at the mitochondria, it is unclear the extent to which Parkin functions in other subcellular compartments. In this dissertation, I demonstrate that a portion of Parkin localizes to the nucleus *in vitro* and *in vivo*. Furthermore,

Parkin's nuclear localization is dynamic; Parkin rapidly exits the nucleus during nutrient deprivation and translocates to the nucleus in response to hypoxia.

To focus on roles of Parkin at distinct subcellular locations, I generated mitochondrial- and nuclear-targeted Parkin constructs. Nuclear-localized Parkin can contribute to mitochondrial-stress-induced mitophagy. Further, I demonstrate widespread transcriptional changes in response to Parkin in the nucleus, especially across a variety of metabolic pathways and pathways regulated by nuclear receptors and nuclear receptor cofactors.

Through a non-biased proteomics screen, I identified the transcription factor estrogen related receptor α (ERR α) as a potential Parkin target. I demonstrate that nuclear-targeted Parkin interacts with ERR α but does not affect its stability or degradation. Instead, expressing nuclear-Parkin promotes transcription of ERR α targets.

Overexpression of Parkin in tissues has been shown to reduce age-associated cardiac dysfunction and preserve mitochondrial function in mice. Although several studies have identified a role for Parkin in acute cardiac stress, whether Parkin plays a role in adaptation to chronic cardiovascular stressors is still unclear. Therefore, I investigate the role of Parkin in a chronic cardiovascular disease model. In hearts from Parkin transgenic mice, mitochondrial Complex II activity was increased in response to chronic pressure-overload. However, Parkin overexpression did not rescue pressure overload-induced cardiac dysfunction or hypertrophy.

My findings reveal an expanded role for Parkin in response to a variety of acute cellular stressors. I identify a novel role for Parkin in mediating transcription of metabolic

genes from the nucleus that could contribute to understanding how mutations in Parkin lead to Parkinson's Disease. Additionally, I verify that although Parkin functions to protect the heart against acute damage, increasing Parkin levels does not protect the heart from chronic pressure-overload.

CHAPTER 1: INTRODUCTION

1.1 Ubiquitination and the E3 Ubiquitin Ligase Parkin

Ubiquitination is a post-translational modification, in which ubiquitin is covalently attached to target proteins at lysine residues, although other amino acids may also be utilized (Yau and Rape, 2016). Ubiquitination requires a series of events involving ubiquitin activation, conjugation, and ligation, which are catalyzed by E1, E2, and E3 enzymes respectively (Figure 1.1 A). When lysine is the residue in the target protein modified by ubiquitin, the chemical linkage is an isopeptide bond between the carboxyl group of the final amino acid of ubiquitin (glycine) and the epsilon amino group of the lysine. In some instances, only a single ubiquitin is added (monoubiquitination). Otherwise, the initial ubiquitin extends into a chain through covalent bonding between the C-terminal glycine of the next ubiquitin with various lysines (or the N-terminal methionine) of the preceding ubiquitin (polyubiquitination).

Ubiquitination can affect a protein in multiple ways, including promoting its degradation through the proteasome or through autophagy, changing its subcellular localization, activity, or interactions with other proteins (Figure 1.1 A). These diverse effects may impact multiple cellular processes including transcription, translation, DNA repair, organelle trafficking, cell survival/death, cell proliferation, and inflammation, to name just a few. While the precise mechanisms by which ubiquitination of a particular protein bring about these effects are incompletely understood, important factors include which residues on the target protein undergo ubiquitination, whether mono- or polyubiquitination is involved, and the nature of the ubiquitin-ubiquitin linkages in the

case of polyubiquitination. For instance, polyubiquitylation often leads to protein degradation, while some instances of monoubiquitination stabilize proteins or change their subcellular locations (Yau and Rape, 2016). In the ubiquitination pathway, the E3 ligases are responsible for much of the target specificity and pathway regulation (Seirafi et al., 2015).

Parkin is one of more than 500 E3 ubiquitin ligases encoded by the human genome, and comprises five distinct domains: Ubiquitin-like (Ubl), Really Interesting New Gene (RING) 0, RING1, In-between-RING (IBR), and RING2 (Figure 1.1 B). Parkin is the most well-studied of the 14 members of the RING-between-RING (RBR) subfamily of E3 ligases. The Ubl and RING0 domains are unique to Parkin, while the RING1-IBR-RING2 domain combination is shared with the remaining RBR ligases (Arkinson and Walden, 2018). The regulation of Parkin and its activity is currently a rapidly-developing field. Recent publications have discovered critical roles for several phosphorylation events in activating Parkin. Until activation, Parkin remains in an autoinhibited conformation in which its Ubl and RING0 domains are folded back to interact with the RING1 and RING2 domains to block the predicted E2 binding site on the RING1 and the catalytic cysteine residue in the RING2 (Caulfield et al., 2015). Parkin is phosphorylated on its Ubl domain at Ser65, which induces a large conformational change to increase its activation (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Tang et al., 2017). Parkin is further activated by the phosphorylation of ubiquitin (Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014). Finally, the phosphorylation of both Parkin and ubiquitin increases the interaction between Parkin and its E2s (Kumar et al., 2015; Sauve et al., 2015). Parkin is an

important regulator in many cellular processes, and understanding how Parkin is regulated helps us to understand both Parkin's functions and how the ubiquitination process is regulated more broadly.

1.2 Parkin and Mitochondrial Quality Control

Mitochondria generate energy for cellular processes through oxidative phosphorylation. Dysfunctional mitochondria are less efficient at generating ATP, produce excessive amounts of reactive oxygen species (ROS), and may activate cell death (Baines, 2010). To ensure a network of functional mitochondria, cells have quality control mechanisms in place that act at both the protein and organelle levels. Damaged mitochondrial proteins are degraded by mitochondrial proteases and the ubiquitin-proteasome system (Goldberg, 2003). If the degradation of damaged proteins is insufficient to rescue the mitochondrion, then the organelle is engulfed by an autophagosome and subsequently delivered to a lysosome for degradation.

The process of autophagy involves a double-membraned autophagosome that engulfs cytoplasm and organelles, and then fuses with a lysosome for degradation (Yang and Klionsky, 2010). The activation of a complex composed of BECLIN 1, vacuolar protein sorting (VPS) 34, and VPS15 leads to nucleation of a double-membrane structure called the phagophore, or isolation membrane (Zeng et al., 2006). Two ubiquitin conjugating systems—Microtubule-associated protein 1 light chain 3 (LC3) (Kabeya et al., 2004) and ATG12-ATG5-ATG16 (Kuma et al., 2002)—are involved in elongation of the membrane. LC3 is also responsible for tethering the autophagosome to the cargo (Lamark et al., 2009; Pankiv et al., 2007). The

autophagosome then encloses around the cargo, fuses with the lysosome, and the contents are degraded by lysosomal enzymes (Yang and Klionsky, 2010). Under normal physiological conditions, basal autophagy maintains homeostasis by degrading long-lived proteins, protein aggregates, and abnormal or excess organelles. Although originally thought to be non-selective, it is now clear that autophagy can selectively target protein aggregates (Tannous et al., 2008) as well as organelles such as peroxisomes (Iwata et al., 2006), endoplasmic reticulum (Bernales et al., 2007), and mitochondria (Elmore et al., 2001). This selective removal of impaired mitochondria by autophagosomes is known as mitochondrial autophagy, or mitophagy. Both autophagy and mitophagy are critical for cellular homeostasis (Billia et al., 2011; Hoshino et al., 2013; Kubli et al., 2013a; Nakai et al., 2007) and adapting to acute unfavorable conditions such as starvation or ischemia (Hamacher-Brady et al., 2006; Kanamori et al., 2009; Matsui et al., 2007).

The most well-characterized pathway involved in regulating mitophagy is the PINK1/Parkin pathway. When mitochondria are healthy, newly-synthesized serine/threonine kinase PTEN-inducible kinase 1 (PINK1) is imported into the mitochondrial matrix by the translocase of the outer membrane (TOM) complex (Lazarou et al., 2012) where it is cleaved by mitochondrial processing proteinase (MPP) and presenilin associated rhombin-like (PARL) (Jin et al., 2010). Therefore, cellular levels of PINK1 are always low at baseline. However, upon loss of mitochondrial membrane potential, PINK1 ceases to be imported and instead accumulates on the outer mitochondrial membrane (Jin et al., 2010). This leads to recruitment of Parkin from the cytosol to the mitochondrial membrane, where it ubiquitinates a host of

mitochondrial proteins (Narendra et al., 2008). Studies have demonstrated that Parkin translocation is dependent on PINK1 (Kane et al., 2014; Koyano et al., 2014; Okatsu et al., 2012b). Several mitochondrial Parkin substrates have been identified and characterized, including hexokinase 1 (Okatsu et al., 2012a), VDAC1 (Geisler et al., 2010), MFN 1 and 2 (Tanaka et al., 2010), and MIRO (Wang et al., 2011). The functional importance of these substrates in Parkin-mediated mitophagy is somewhat controversial. Studies indicate that there is a redundancy between substrates, and that the loss of one substrate has little or no effect on Parkin-mediated mitophagy. It is not surprising that this redundancy exists because it ensures clearance of mitochondria even if one substrate is downregulated or mutated. Studies also suggest that Parkin-mediated protein ubiquitination serves two purposes to facilitate mitophagy. Ubiquitination of mitochondrial proteins via Lys63-linkage marks them for degradation by the autophagosome. Adaptor proteins such as p62 and NBR1 bind to LC3 on the autophagosome and to the ubiquitinated mitochondrial proteins, tethering the autophagosome to the mitochondrion (Kirkin et al., 2009; Lamark et al., 2009). Ubiquitination of mitochondrial fusion proteins MFN1/2 via Lys48-linkage leads to their degradation by the ubiquitin proteasome system (Tanaka et al., 2010). This causes a shift in the balance between mitochondrial fission and fusion towards fission. Fission increases the probability of the mitochondrial fragment being removed by mitophagy (Gomes and Scorrano, 2008; Twig et al., 2008). It is likely that there are many additional Parkin substrates, both at the mitochondria and in other subcellular compartments that are currently unknown.

1.3 Parkin's Role in Disease

1.3.1 Parkinson's Disease

Parkinson's Disease (PD) affects approximately 1-2% of individuals over age 60 and up to 4% of the population older than 85 (Fahn, 2003; Ibanez et al., 2017). Symptoms include a variety of motor and neurological features including resting tremor, bradykinesia, rigidity, postural instability, olfactory dysfunction, cognitive impairment, psychiatric symptoms, and autonomic dysfunction. On the cellular level, PD is characterized by loss of dopaminergic neurons, primarily located within the substantia nigra, often accompanied by intracellular inclusions termed Lewy bodies. Currently, the mechanism for dopaminergic neuron loss is not well understood. The proposed model for pathogenesis includes protein misfolding and aggregation, mitochondrial injury, energetic failure, oxidative stress, and inflammation (Del Rey et al., 2018). Although most cases of PD are sporadic, mutations in the *PRKN* gene are the most common cause of autosomal recessive early onset PD (Ferreira and Massano, 2017). Through extensive research, there are several treatments to ameliorate symptoms of PD in patients, however, there are no known cures (Del Rey et al., 2018).

Although it is clear that mutations that impair Parkin's E3 ligase function contribute to the attrition of dopaminergic neurons through cell death in Parkinson's disease (Pickrell and Youle, 2015), the underlying mechanisms are still not fully understood and there is no direct evidence that a defect in mitophagy is the underlying cause of Parkinson's disease (Scarffe et al., 2014). The assumption that faulty mitophagy is responsible for Parkinson's disease stems from observations that defects in mitochondrial morphology and function are key features of Parkinson's disease (Pickrell and Youle, 2015) and that loss of Parkin in *Drosophila* leads to mitochondrial

dysfunction and muscle degeneration (Greene et al., 2003). When combined with the seminal work from the Youle lab demonstrating that Parkin can mediate mitophagy (Narendra et al., 2008), it is easy to see how this model—where defects in mitophagy due to loss of Parkin function cause accumulation of dysfunctional mitochondria and Parkinson's disease—was generated. However, Parkin is not only activated during mitochondrial depolarization; Parkin protects neurons against a variety of cellular stressors including endoplasmic reticulum stress (Takahashi et al., 2003), proteasomal dysfunction (Tsai et al., 2003), manganese-induced cell death (Higashi et al., 2004), and oxidative stress (Hyun et al., 2005). Additionally, brain homogenates from Parkin-null mice still maintain functional mitochondria in the absence of additional stressors (Pickrell et al., 2015). Therefore, either alternative mitochondrial quality control mechanisms exist to compensate for Parkin deficiency, or maintaining mitochondrial homeostasis under baseline conditions is not Parkin's principal function, and therefore not the primary cause of pathology in Parkinson's disease.

1.3.2 Cardiovascular Disease

Approximately one out of every three deaths in the US can be attributed to heart disease, and the US spends more money on diagnosing and treating heart disease than on any other type of disease (Benjamin et al., 2019). As the incidence of heart disease increases with age and the population ages as a whole, both the prevalence of heart disease and the annual expenditure are expected to increase substantially (Benjamin et al., 2019).

During the development of heart failure, the heart loses cardiac myocytes – the cells responsible for maintaining cardiac contractility. With fewer cells, the heart is unable to sustain contraction, and patients develop symptoms of heart failure such as edema, fatigue, and weakness (Friedman and Quinn, 2008). Similar to PD, there are treatments for the symptoms of heart failure, but no cure. Thus, understanding the underlying mechanisms of myocyte loss is critical. The heart is densely packed with mitochondria responsible for generating the ATP necessary for the beating cardiomyocytes. Similar to other tissues in the body, defective or damaged mitochondria can become harmful to myocytes by generating excessive reactive oxygen species and releasing pro-death proteins, causing unnecessary cell death. Myocyte death is especially harmful as myocytes are post-mitotic and cannot be replaced.

Impaired mitophagy in the heart leads to accumulation of aberrant mitochondria and subsequent contractile dysfunction (Billia et al., 2011; Hoshino et al., 2013; Kubli et al., 2013a). Recent studies have demonstrated that loss of Parkin also has negative consequences for the heart. Similar to their lack of neurological phenotype, germline Parkin-null mice have normal cardiac function when young (Kubli et al., 2013a; Kubli et al., 2013b). Likewise, deleting Parkin specifically from adult myocytes has few functional consequences for mitochondria or cardiac function (Song et al., 2015). Therefore, it is likely that under baseline conditions, myocytes have sufficient alternative mitophagy mechanisms to maintain mitochondrial homeostasis in the absence of Parkin. However, when myocytes experience stress or an increased need for mitochondrial turnover, Parkin appears to be critical. For example, Parkin-null mice accumulate abnormal mitochondria with age (Kubli et al., 2013a). Additionally, Parkin is required for removal

of cardiac mitochondria during the switch from glucose to fatty acid metabolism in early postnatal life (Gong et al., 2015). In response to myocardial infarction, Parkin-deficiency leads to development of heart failure, and increased mortality (Kubli et al., 2013a). Doxorubicin is a chemotherapeutic associated with cardiotoxicity and mitochondrial damage in myocytes (Zhou et al., 2001). A recent study has linked the doxorubicin's cardiotoxicity to impaired Parkin translocation to mitochondria, resulting in diminished clearance of dysfunctional mitochondria (Hoshino et al., 2013). Therefore, although alternative mechanisms of mitophagy may be sufficient to compensate for loss of Parkin under normal homeostatic conditions, Parkin is necessary for responding to certain cardiac stress conditions.

1.3.3 Cancer

Although Parkin has generally been portrayed as a neuroprotector, several recent studies suggest that Parkin also functions as a tumor suppressor (Cesari et al., 2003; Denison et al., 2003; Mehdi et al., 2011). Genetic mutations or alterations in Parkin expression have been found in a variety of tumor biopsies and tumor cell lines from an assortment of cancers, including breast, ovarian, skin, thyroid, bladder, pancreatic, esophageal, and lung cancers (Cesari et al., 2003; Denison et al., 2003; Mehdi et al., 2011; Wang et al., 2004; Xu et al., 2014). Interestingly, overexpressing Parkin has been shown to inhibit cell growth in hepatocarcinoma (Wang et al., 2004), breast cancer (Tay et al., 2010), glioblastoma (Veeriah et al., 2010), and colorectal cancer (Poulogiannis et al., 2010) cell lines. In contrast, knocking down Parkin promotes proliferation and tumorigenesis in pancreatic cancer (Sun et al., 2013), and Parkin-null

mice develop hepatocarcinoma and intestinal polyps with age (Fujiwara et al., 2008; Lee et al., 2019). Although loss of Parkin function is associated with several tumorigenic processes including activating invasion and metastasis, resisting cell death, and deregulating cellular energetics, many of the known targets and mechanisms relate to de-regulation of the cell cycle (Xu et al., 2014). For instance, Parkin mediates the degradation of Cyclins D and E, which both function to promote cell cycle progression (Ikeuchi et al., 2009; Yeo et al., 2012). Parkin also interacts with Cdc20 and Cdh1 in the anaphase-promoting complex/cyclosome to increase degradation of proteins known to regulate mitosis (Lee et al., 2015). Taken together, these data reveal that, although it is clear that inactivation of the *PRKN* gene is a frequent event in many cancer types, specific roles and ubiquitination targets of Parkin relevant to cancer are largely uncharacterized. Further research is needed to expand upon our knowledge of the regulation of Parkin, its mechanisms of action, and novel targets, both at the mitochondria and elsewhere.

1.4 Non-mitophagy Functions of Parkin

The fact that numerous cell lines, such as mouse embryonic fibroblasts and HeLa cells, have undetectable levels of Parkin under normal conditions and still maintain networks of healthy mitochondria indicates that Parkin is not crucial for mitochondrial homeostasis. As an E3 ubiquitin ligase, Parkin function has the potential to impact numerous pathways/processes beyond mitochondrial quality control. Indeed, an unbiased proteomic analysis identified a broad range Parkin substrates - even when performed within the relatively circumscribed context of experimentally induced

mitochondrial damage (Sarraf et al., 2013). Members of the Parkin ubiquitylome identified in this study included several mitochondrial proteins, as expected. Several other proteins such as HMGB1, MED17 and NF κ B were also identified to be potential Parkin substrates. The functions of the proteins identified mapped to several distinct pathways including autophagy, proteasome function, mitochondrial function, mitochondrial dynamics, cell death, and metabolism. Many of the proteins from this study, however, have yet to be confirmed as true Parkin targets.

Recent studies have begun identifying non-mitophagy targets and functions of Parkin. For example, in addition to mediating mitophagy, Parkin also activates mitochondrial biogenesis by ubiquitinating Parkin-interacting substrate (PARIS) for degradation (Shin et al., 2011). Because PARIS transcriptionally represses mitochondrial biogenesis regulator peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1 α), Parkin-mediated degradation of PARIS increases mitochondrial biogenesis. Additionally, Parkin regulates lipid metabolism by stabilizing the plasma membrane CD36 lipid transporter, which also causes Parkin-deficient mice on high-fat diet (HFD) to withstand weight gain, steatohepatitis, and insulin resistance (Kim et al., 2011). Parkin has also been implicated in the regulation of cell death. Parkin inhibits the mitochondrial apoptosis pathway by ubiquitinating Bax, prohibiting its translocation from the cytosol to mitochondria in response to apoptotic stimuli (Johnson et al., 2012; Sarraf et al., 2013). Parkin may conversely sensitize cells to apoptosis induced by mitochondrial membrane depolarization, but not by classic mitochondrial and death receptor apoptosis activators, via ubiquitination and degradation of the anti-apoptotic Bcl-2 protein, Mcl-1 (Carroll et al., 2014).

1.5 Rationale of the Thesis

Parkin plays a role in a variety of diseases, including Parkinson's Disease, heart disease, and cancer, yet mechanisms and consequences of Parkin's functions are still incompletely understood. The repertoire of Parkin substrates remains poorly characterized, and to date, the majority of Parkin substrates are mitochondrial proteins. However, recent studies have begun to uncover novel, non-mitophagy functions for Parkin. Nonetheless, the diversity and complexity of Parkin's functions extend beyond mitophagy, and our current understanding of Parkin's role health and disease needs to be reevaluated and expanded. As an E3 ubiquitin ligase, Parkin has the potential to impact numerous pathways and processes beyond mitophagy. In this thesis, I provide new insight into the functional role of Parkin in the nucleus.

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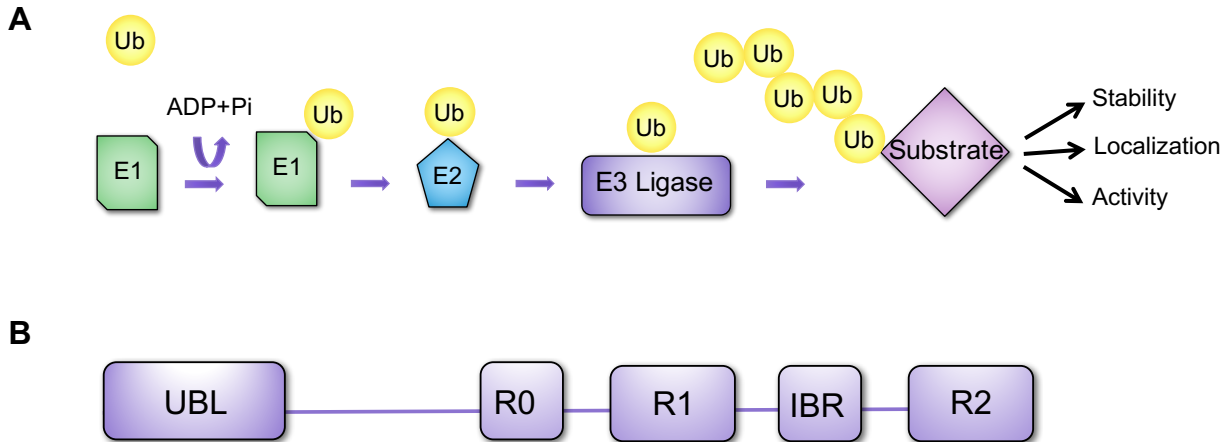


Figure 1.1 Diagram of Ubiquitination and the E3 Ubiquitin Ligase Parkin. A) Ubiquitin is activated by an E1 enzyme, conjugated by an E2, and then transferred from to a substrate protein either directly or indirectly through the use of an E3 ubiquitin ligase. Substrate proteins can be either mono- or poly-ubiquitinated, and ubiquitination can promote a change in a substrate's stability, localization, or activity. B) Parkin comprises five domains: Ubiquitin like (UBL), Really Interesting New Gene 0 (RING0, R0), RING1 (R1), in-between-ring (IBR), and RING2 (R2).

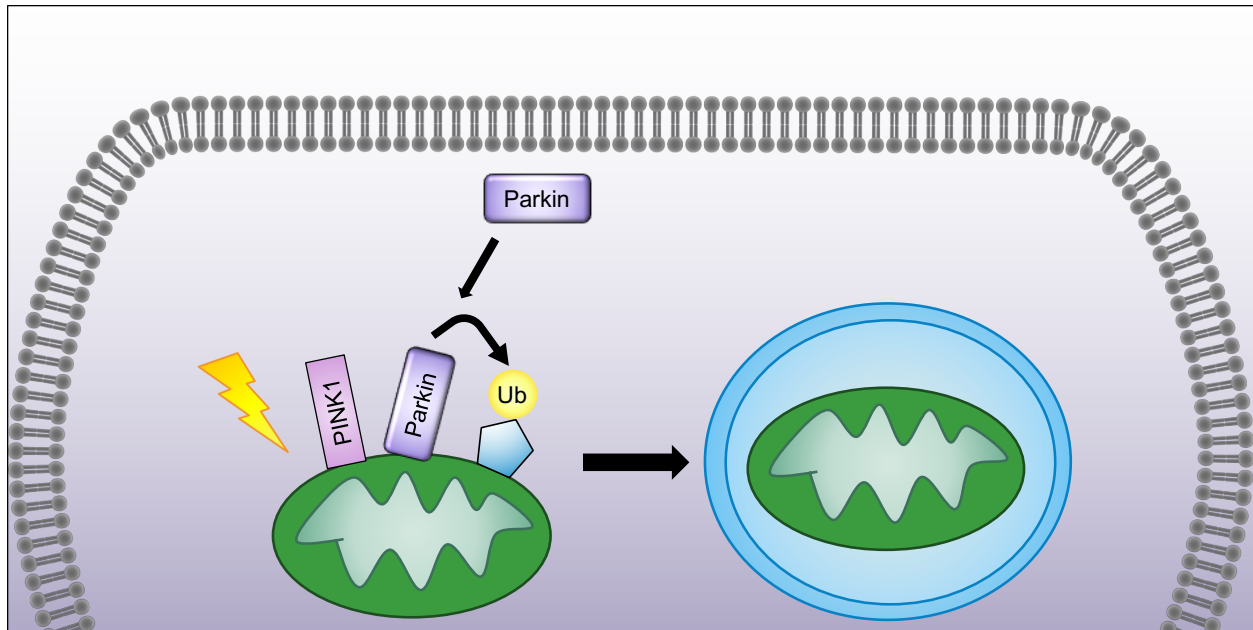


Figure 1.2. Diagram of Parkin-mediated Mitophagy. Upon mitochondrial damage, kinase PINK1 is stabilized on the outer mitochondrial membrane, which recruits Parkin from the cytosol to the outer mitochondrial membrane where it ubiquitinates several proteins that mark the mitochondria for sequestration by an autophagosome and degradation through fusion with a lysosome.

CHAPTER 2: SUBCELLULAR LOCALIZATION OF PARKIN AT BASELINE AND IN RESPONSE TO STRESS

2.1 Introduction

PD-associated mutants of Parkin are spread throughout all of Parkin's domains as well as in linkers in between domains (Fiesel et al., 2015). Mutations may affect Parkin's ligase function, subcellular localization, or cause protein misfolding and aggregation (Fiesel et al., 2015; Tan et al., 2009; Winklhofer et al., 2003; Wong et al., 2007). Accumulating evidence suggests that not only does Parkin function at the mitochondria, but that it also has substrates in other cellular compartments. Therefore, altering Parkin's subcellular localization may limit its access to substrates or change which substrates it ubiquitinates. Researchers have focused on determining the mechanism by which PD-mutants affect Parkin function and disease pathology.

Parkin is highly regulated by post-translational modification. Parkin activation and translocation to the mitochondria are both promoted by PINK1-mediated phosphorylation (Panicker et al., 2017). There are three distinct phosphorylation events that cause Parkin's activation and translocation to mitochondria. PINK1 phosphorylates the Ubl domain of Parkin at Ser65, PINK1 phosphorylates ubiquitin at Ser65, and PINK1 phosphorylates proteins on the mitochondria that act as Parkin receptors (Chen and Dorn, 2013; Okatsu et al., 2015). Whether there is a single, or many Parkin receptors on the mitochondria is uncertain (Dorn, 2016a). One study shows that PINK1-mediated phosphorylation of Mfn2 is required for Parkin mitochondrial localization (Chen and Dorn, 2013). Another group, instead, reports that phosphorylated ubiquitin chains are

the Parkin receptor on the mitochondria (Okatsu et al., 2015). It is probable, however, that a degree of functional redundancy exists in regulating Parkin's translocation to the mitochondria as it is critical in responding to mitochondrial stress (Dorn, 2016a). Because Parkin's localization is dynamic and cellular-stress-dependent, I hypothesized that Parkin may localize to additional cellular compartments during non-mitochondrial stress conditions.

The mechanisms of Parkin's ligase activity and their consequences on physiology and disease states are studied using a variety of different models. Studies parsing out Parkin's function in the mitochondria-rich heart are mainly executed using mouse models (Dorn, 2016b). Although convenient for examining the effects of losing Parkin function, a major limitation of current Parkin knockout mouse models is that they fail to replicate the PD phenotype, likely due to compensation by other E3 ligases (Bhandari et al., 2014). Alternatively, many of the studies examining mutations in Parkin and how they affect protein folding, binding, and function are conducted on the cellular and molecular level. Much of the recent progress made in determining signaling mechanisms of Parkin has been in cell lines overexpressing fluorescently-tagged Parkin constructs, as many immortalized cell lines, do not endogenously express Parkin (Ding et al., 2010). In this chapter, I utilize fluorescently-tagged Parkin constructs in cell lines that lack endogenous Parkin to extensively explore Parkin subcellular localization, and I examine how PD-associated mutations and various cellular stressors alter its localization.

2.2 Results

2.2.1 Parkin Localizes to the Nucleus in vitro

To determine how pathologic mutants of Parkin differ from wild type (WT) Parkin, we first examined their localization. When overexpressed in HeLa cells under baseline conditions, mCherry-tagged Parkin localizes throughout the cell (Figure 2.1 A and B). However, pathogenic mutants of Parkin, ParkinR42P and ParkinG430D, are excluded from the nucleus. To confirm this is not a cell-type specific phenomenon, we repeated the experiment in mouse embryonic fibroblasts (MEFs) and neonatal rat ventricular myocytes (NRVMs) with similar results (Figure 2.1, C-F). Notably, the Parkin mutants did not express well in the NRVMs, and the subcellular localization of ParkinG430D was not able to be quantified. Therefore, we examined whether expression of ParkinR42P and ParkinG430D is toxic through a cell death assay (Figure 2.2). We did, indeed, find that expression of both Parkin mutants is cytotoxic. Together, these data suggest that Parkin is not solely a cytosolic E3 ligase under baseline conditions; but that a portion of it also exists in the nucleus. Additionally, PD-associated mutants of Parkin are selectively excluded from the nucleus, and are cytotoxic.

2.2.2 Parkin Localizes to the Nucleus in vivo

Next, I examined whether Parkin also localizes to the nucleus in vivo. I harvested hearts from Parkin^{-/-} (Kubli et al., 2013b), WT, and cardiac-specific Parkin transgenic (TG) mice (Woodall et al., 2019) and performed a subcellular fractionation and western blotting of nuclear and cytosolic fraction lysates. In the Parkin TG hearts, I detected the majority of Parkin in the cytosolic fraction, while a portion of Parkin existed in the nucleus (Figure 2.3 A). Additionally, when I probed the immunoblots with an antibody

against ubiquitin, I found an increase in total nuclear protein ubiquitination in the crude nuclear fractions from the Parkin TG hearts compared to the WT hearts (Figure 2.3 B). These data suggest that, not only does a portion of Parkin exist in the nucleus *in vivo*, but that it may also have an active role in ubiquitinating nuclear proteins.

Next, I verified that endogenous Parkin also localizes to the nucleus. Although Parkin protein is detectable in most cells, tissues, and organs throughout the body, it is present at higher levels in highly-metabolically active tissues such as the heart, brain, and liver (Huynh et al., 2001). To confirm the nuclear localization of WT Parkin, I performed subcellular fractionation of a variety of tissues that express Parkin from WT mice (Figure 2.3 C). Similar to Parkin in the transgenic mouse hearts, the majority of endogenous Parkin protein was present in the cytosolic fraction, but a portion was detected in the nuclear fraction as well. Therefore, I concluded that a portion of endogenous Parkin localizes to the nucleus in multiple tissues and cell types.

2.2.3 Parkin Translocates to the ER in Response to Thapsigargin Treatment

Parkin is canonically characterized as localizing to the cytosol under baseline conditions and translocating to damaged mitochondria in response to mitochondrial depolarization (Narendra et al., 2008). Therefore, I examined whether Parkin's localization in other cellular compartments is similarly dynamic, and if so, under what conditions. To do this, I subjected cells to a variety of stressors and examined Parkin's localization via immunofluorescence and western blotting. Parkin is protective during endoplasmic reticulum (ER) stress (Han et al., 2017). To determine whether Parkin changes localization in response to ER stress, I treated HeLa cells expressing mCherry-

Parkin with Thapsigargin. Thapsigargin inhibits the Sarco/endoplasmic reticulum calcium ATPase (SERCA), which normally pumps calcium from the cytoplasm back into the ER (Thastrup et al., 1990). When SERCA is inhibited, calcium accumulates in the cytoplasm and is depleted from stores in the ER, causing ER stress. Interestingly, in response to Thapsigargin treatment, I found that Parkin co-localizes with ER-marker, Calnexin (Figure 2.4). This suggests that Parkin translocates to the ER during ER-related stress.

2.2.4 Parkin Rapidly Exits the Nucleus in Response to Nutrient Deprivation

Starvation is a potent activator of general autophagy but not Parkin-mediated mitophagy. I therefore tested whether starvation alters Parkin's subcellular localization. To do this, I deprived HeLa cells stably expressing YFP-Parkin of serum and glucose for up to one hour. Using immunofluorescence, I found that Parkin rapidly exits the nucleus in as few as 15 minutes, in response to nutrient deprivation (Figure 2.5 A and B). To further confirm the findings, I performed a subcellular fractionation of cells harvested under the same conditions and immunoblotted for Parkin in the crude nuclear and cytosolic fractions (Figure 2.5 C-D). Indeed, during nutrient deprivation I detected a decrease in Parkin in the nuclear fraction as compared to the amount of Parkin in the cytosolic fraction, suggesting that Parkin rapidly exits the nucleus in response to nutrient deprivation. These data indicate that Parkin nuclear localization appears to be dynamic.

2.2.5 Parkin Translocates to the Nucleus in Response to Hypoxia Independent of PINK1

Hypoxia reduces cellular energy production through decreasing mitochondrial respiration and reducing mitochondrial biogenesis through HIF1 α and PGC1 α signaling (Wu and Chen, 2015). Energetic imbalances can, in turn, contribute to both neurodegenerative disease and heart disease. Therefore, I assessed whether hypoxia alters Parkin's localization. Similar to previous experiments, I used YFP-Parkin HeLa cells and subjected them to hypoxia for up to 24 hours. I detected increased Parkin in the nucleus in response to hypoxia within 24 hours by immunofluorescence (Figure 2.6 A and B). To further confirm the findings, I performed a subcellular fractionation on cells expressing mCherry-Parkin and harvested under the same conditions. Confirming my immunofluorescence data, I detected increased Parkin in the crude nuclear fraction in response to hypoxia (Figure 2.6 C and D). Collectively, these data suggest that Parkin translocates to the nucleus in response to hypoxia.

To assess whether Parkin is active while localized to the nucleus, I additionally immunoblotted for ubiquitin in the nuclear and cytosolic fractions. Total nuclear protein ubiquitination was increased in the nucleus and decreased in the cytosol, correlating with amount of Parkin. This suggests that Parkin may ubiquitinate nuclear proteins in response to hypoxia (Figure 2.6 C).

PINK1 is necessary for Parkin activation and translocation to the mitochondria (Kim et al., 2008; Sha et al., 2010; Shiba-Fukushima et al., 2012). It is currently unknown whether PINK1 is also critical for activating Parkin to ubiquitinate non-mitochondrial substrates. Because my previous data indicates that Parkin's localization the nucleus is also dynamic, I determined whether PINK1 is necessary for Parkin to translocate to the nucleus through the use of PINK1-null HeLa cells. Confirming our

previous data, overexpressed mCherry-Parkin translocated to the nucleus in the control HeLa cells in response to hypoxia (Figure 2.7 A). In PINK1-null HeLa cells, I measured an increase in Parkin localizing to the nucleus in response to hypoxia at a similar rate to the control cells (Figure 2.7 B and C). These data indicate that Parkin translocates to the nucleus independently of PINK1 in response to hypoxia.

2.2.6 We did not Identify an Endogenous Nuclear Localization Signal for Parkin

Proteins can localize to the nucleus through several mechanisms. Proteins smaller than approximately 40 kDa can pass through nuclear pore complexes through passive diffusion (Depping et al., 2015). Parkin is 52 kDa and too large to passively diffuse; therefore, I investigated what mediates Parkin's translocation into the nucleus.

The main determinant of nuclear localization is the presence, or absence, of a nuclear localization signal (NLS). To determine whether Parkin contains an NLS, I analyzed the sequence for the presence of NLS sequences or motifs. One potential NLS was identified that spanned residues 408 to 428. To test whether this was a true nuclear localization signal, I used two distinct methods: 1. I linked the putative NLS to the C-terminus of GFP (GFP-NLS) and measured whether it promoted the nuclear localization of GFP, and 2. I generated two different Parkin constructs with mutations in the putative NLS and measured whether it reduced Parkin's nuclear localization at baseline or translocation in response to hypoxia (Figure 2.8 A).

First, I assessed whether the putative NLS altered GFP's nuclear localization by expressing the constructs in HeLa cells and visualizing the nuclear signal via immunofluorescence (Figure 2.8 B and C). Because there appeared to be subtle

changes in nuclear localization versus simple presence or absence of nuclear GFP signal, I measured the ratio of nuclear to cytoplasmic relative fluorescence intensity via ImageJ, but I did not detect any differences between GFP control and GFP-NLS. I additionally confirmed this result via subcellular fractionation and immunoblotting for GFP in both the nuclear and cytosolic fractions (Figure 2.8 D and E).

Next, I determined whether mutating the putative NLS would inhibit Parkin's nuclear localization both at baseline, and in response to hypoxia. Similar to above, I overexpressed WT and mutant Parkin in HeLa cells and measured nuclear signal at baseline and in response to hypoxia via immunofluorescence (Figure 2.8 F). When I quantified pixel intensity as above, I did not detect a difference in nuclear localization between WT Parkin and the mutants (Figure 2.8 G). I additionally confirmed this result via subcellular fractionation and immunoblotting for Parkin in both the nuclear and cytosolic fractions (Figure 2.8 H-J). Collectively, these data indicate that we could not identify an endogenous NLS for Parkin.

2.3 Discussion

The mechanism by which loss of Parkin function causes PD is currently unknown. Parkin's roles at the mitochondria have been well-characterized, but whether it functions in other subcellular locations has been unclear. Here, I report that Parkin localizes throughout the cell under baseline conditions, but that PD-associated mutants are excluded from the nucleus. Previous studies have made attempts at examining endogenous Parkin subcellular localization via immunohistochemistry in brains using study-specific antibodies with limited success (Horowitz et al., 2001; Stichel et al.,

2000). In this study, we utilized fluorescently-tagged constructs to discern differences in localization between WT and mutant Parkin in three different cell types.

Parkin-null mice do not develop a PD phenotype, most likely due to compensation by other E3 ligases (Bhandari et al., 2014). Having a mutation in Parkin is therefore functionally different than lacking Parkin all together, which is why the mislocalization of mutated Parkin may be especially relevant to disease pathology. PD-associated mutations occur across every domain of Parkin, which indicates that the structural conformation and cooperation of the domains is crucial to Parkin's functions and development of disease. Previous work has examined point mutations in the RING1 domain of Parkin showing that some aggregate (Cookson et al., 2003). Other studies have assessed how mutations affect Parkin's ability to be phosphorylated or its E3 ligase function (Aguirre et al., 2018; Arkinson and Walden, 2018; Fiesel et al., 2015). The R42P mutation is located in the Ubl domain, which plays a large role in stabilizing Parkin's inactive conformation. The Ubl domain also contains Ser65, which is phosphorylated by PINK1 to activate Parkin. Therefore, conformational changes due to the R42P mutation likely affect Parkin's stability and activation. Alternately, the G430D mutation in Parkin is adjacent to the catalytic cysteine residue, C431, which may affect Parkin's E3 ligase activity. Interestingly, Parkin autoubiquitination has been reported to both positively and negatively regulate its activity (Chakraborty et al., 2017). Thus, it is possible that both phosphorylation of Parkin and its E3 ligase activity are necessary for its nuclear localization.

One question raised by my findings is how Parkin moves from one subcellular compartment to another. I did not identify an endogenous NLS for Parkin; however,

Parkin is widely post-translationally modified, so it is possible that its subcellular localization is regulated through modification. One study claims that S-nitrosylation of Parkin inhibits Parkin's nuclear localization and ability to bind to the p53 promoter (Sunico et al., 2013). Another group has shown that Parkin interacts with SUMO1, and the interaction may promote its nuclear localization (Um and Chung, 2006). Future studies will be directed at whether post-translational modification of Parkin contributes to its dynamic nuclear localization.

Because Parkin has been primarily studied for its role at the mitochondria, much of the previous research has utilized mitochondrial uncoupling agents to “activate” Parkin, artificially targeting it to the mitochondria (Shires et al., 2017). In this study, I used cellular stressors that are more physiological to determine Parkin localization. Through this study, it has become clear that Parkin is a stress-response protein; its subcellular localization and activation are both dependent on cellular conditions.

My findings in this chapter characterize Parkin's nuclear localization as well as provide physiological relevance for what cellular conditions activate Parkin to translocate to and from the nucleus.

2.4 Materials and Methods

Animals

All animal experiments were performed according to guidelines specified by the University of California, San Diego Institutional Animal Care and Use Committee. Parkin KO mice were obtained from Jackson Laboratories (B6. 129S4-Park2tm1Shn/J, stock number 006582) and their cardiac phenotype has been characterized previously (Kubli

et al., 2013b). The Parkin transgenic (TG) mice overexpress human Parkin under the cardiomyocyte-specific α -myosin heavy chain promoter and were generated at the UCSD Mouse Transgenic Core as described previously (Woodall et al., 2019).

Cell Culture

MEFs and HeLa cells were plated and maintained in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher) containing 10% fetal bovine serum (FBS, ThermoFisher) and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), and cultured in 5% CO₂ atmosphere at 37°C. Neonatal rat ventricular myocytes (NRVMs) were prepared via enzymatic digestion of hearts from 1- to 2-day-old Sprague-Dawley rats and plated in a 4:1 ratio DMEM to M199 (Life Technologies) plus 10% FBS and antibiotics. The YFP-Parkin HeLa cell line was a gift from Dr. Albert La Spada, Duke University School of Medicine (Durham, North Carolina) (Gilmore-Hall et al., 2019). The PINK1-null HeLa cells were given to us by Dr. Richard Youle (Nezich et al., 2015). Hypoxia was carried out by replacing culture media with media supplemented with 20mM HEPES pH 7.4 (ThermoFisher), placing culture dishes into BD GasPak EZ pouch (BD Biosciences) filled with nitrogen, and returning them to the incubators. Nutrient deprivation was carried out by aspirating culture media, washing twice with PBS, and replacing with DMEM without glucose (ThermoFisher) or antibiotics. For thapsigargin treatment, cells were treated with 100 nM thapsigargin 24 after infection, and fixed at the time points indicated.

Adenoviral Constructs

We generated the mCherry-Parkin virus from the plasmid (Addgene #23956). mCherry-ParkinR42P and mCherry-ParkinG430D adenoviruses were generated by site-directed mutagenesis as previously described (Kubli et al., 2013b). Cells were infected with adenoviruses in DMEM + 2% heat-inactivated serum and rescued with their respective culture media. Treatments and experiments were performed 24 hrs post-infection.

Cell Death Assay

Cell death was performed as previously described (Hamacher-Brady et al., 2007). Increased plasma membrane permeability to YoPro-1 (Invitrogen) was determined via fluorescence microscopy using a Carl Zeiss AxioObserver Z1 at 10X magnification.

Subcellular Fractionation

Tissues were harvested and flash frozen in liquid nitrogen. Frozen tissues were then pulverized and homogenized in a dounce tissue grinder with 1 mL of isolation buffer (250mM sucrose, 5mM KH₂PO₄, 2mM MgCl₂, 10mM MOPS, 1mM EGTA, 0.1% fatty-acid free BSA, pH7.4) and Complete protease inhibitor (Roche Applied Bioscience). Lysates were cleared through a 10-minute centrifugation at 200 x g. Supernatant was centrifuged at 800 x g for 15 minutes. The pellet, which makes up the crude nuclear fraction, was washed with 500 µL of isolation buffer, resuspended in 200 µL RIPA buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, and Complete protease inhibitor and incubated on ice for 30 minutes. The supernatant, the crude cytosolic fraction, was

centrifuged at max speed for 15 minutes. Both nuclear and cytosolic fractions were centrifuged at max speed for 5 minutes and transferred to a new tube.

HeLa cells were scraped in cold PBS, centrifuged at 500 x g for 5 minutes and resuspended in buffer containing 10mM HEPES pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, 0.2% NP40. After 15 minutes of incubation on ice on an agitator at 70 rpm, the cytosolic fraction was taken as the supernatant from a 5-minute, 22,000 x g centrifugation. The pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Complete protease inhibitor, and incubated on an agitator at 70 rpm on ice for 15 minutes, and centrifuged for 5 minutes at 2,800 x g. The supernatant was removed and labeled the mitochondrial fraction. The pellet was resuspended in RIPA buffer as the nuclear fraction. All fractions were centrifuged at 22,000 x g for 3 minutes and transferred to a new tube.

Immunoblotting

HeLa cells were harvested and lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Complete protease inhibitor (Roche Applied Science). Samples were run on NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were immunoblotted with the following antibodies: Parkin (Cell Signaling Technology), Histone H3 (Cell Signaling Technology), Lamin A/C (Cell Signaling Technology) GAPDH (GeneTex), Ubiquitin (Santa Cruz Biotechnology) and imaged using a ChemiDoc XRS+ System (Bio-Rad).

Fluorescence Microscopy

Cells were fixed, permeabilized, and blocked as described previously (Hammerling et al., 2017). The cells were stained with anti-TOMM20 (Santa Cruz Biotechnology, 11415) to label mitochondria and incubated with Alexa Fluor 488 or 594 secondary antibodies (Life Technologies A11037) and Hoechst 33342 (Life Technologies, H3570) to stain nuclei. Cells were imaged using a Carl Zeiss AxioObserver Z1 fitted with a motorized Z-stage. Z-stacks were acquired in ApoTome mode using a high-resolution AxioCam MRm digital camera, a 63X Oil-immersion objective and Zeiss AxioVision 4.8 software (Carl Zeiss). For visualizing Parkin nuclear translocation images were taken on the nuclear plane, and presence or absence of Parkin was counted for a minimum of 100 cells per experimental replicate, unless otherwise noted. For quantifying nuclear Parkin in NLS-mutants, the mean fluorescence intensity ratio of nuclear vs cytosolic signal was determined and quantified using Intensity Ratio Nuclei Cytoplasm Tool on ImageJ.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Graphed data were represented as mean $\pm$ SEM. Unpaired Student's t test was used for two-group comparisons. One- or two-way ANOVA with post hoc comparisons by Tukey's method was used for the comparisons between multiple groups.

Parts of Chapter 2 were submitted for publishing. Shires, S. E., Quiles, J. M., Najor, R. H., Leon, L. J., Cortez, M. Q., Lampert, M. A., Mark, A., Gustafsson, Å. B. Nuclear Parkin Promotes the ERRA-mediated Transcriptional Program and Drives Widespread Changes in Gene Expression Following Hypoxia. The dissertation author was the primary investigator and author of this paper.

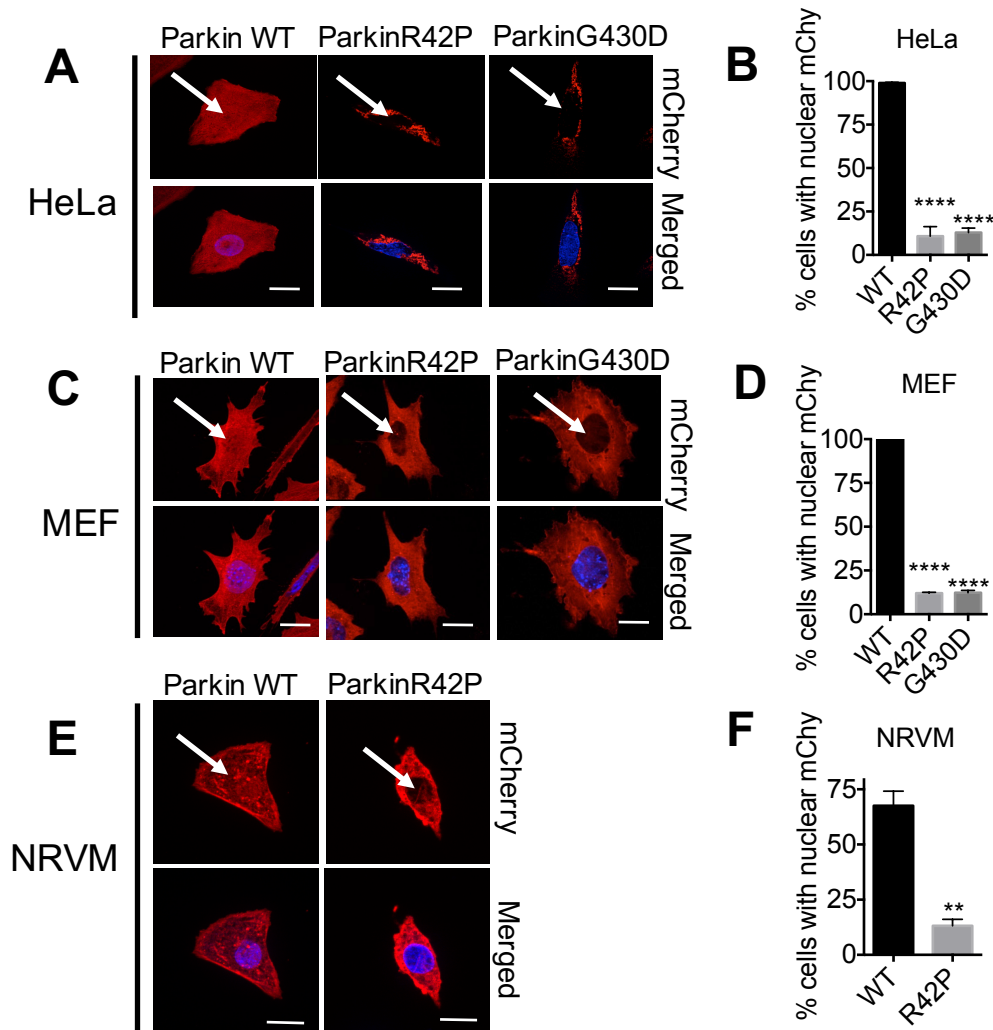


Figure 2.1. Parkin Localizes to the Nucleus in vitro. A) Representative fluorescence microscopy images of HeLa cells expressing mCherry-Parkin WT, mCherry-ParkinR42P, or mCherry-ParkinG430D. B) Quantification of presence or absence of Parkin in the nucleus of HeLa cells expressing the adenoviruses labeled. A minimum of 100 cells were counted for n=3 experimental replicates. C) Representative fluorescence microscopy images of MEFs expressing mCherry-Parkin WT, mCherry-ParkinR42P, or mCherry-ParkinG430D. D) Quantification of presence or absence of Parkin in the nucleus of MEFs expressing the adenoviruses labeled. A minimum of 100 cells were counted for n=3 experimental replicates. E) Representative fluorescence microscopy images of NRVMs expressing mCherry-Parkin WT, mCherry-ParkinR42P, or mCherry-ParkinG430D. F) Quantification of presence or absence of Parkin in the nucleus of NRVMs expressing the adenoviruses labeled. A minimum of 40 cells were counted for n=3 experimental replicates. Scale bars represent 20 μ m for HeLa cells and MEFs and 10 μ m for NRVMs. **p<0.01, ****p<0.0001 compared to WT Parkin.

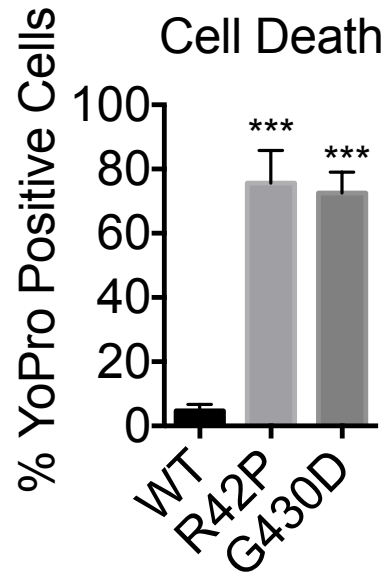


Figure 2.2. PD-associated Parkin Mutants are Cytotoxic. Quantification of cell death in HeLa cells expressing mCherry-Parkin WT, mCherry-ParkinR42P, or mCherry-ParkinG430D. A minimum of 100 cells were counted for n=3 experimental replicates. ***p<0.001 compared to WT Parkin.

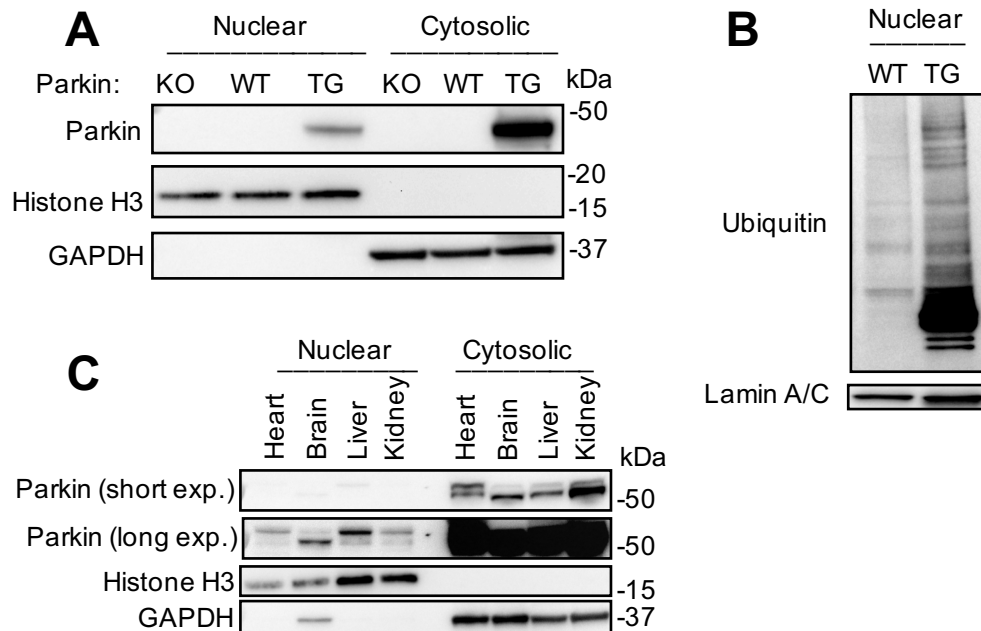


Figure 2.3. Parkin Localizes to the Nucleus in vivo. A) Representative subcellular fractionation and immunoblot for Parkin in nuclear and cytosolic fractions from heart lysates of Parkin KO, WT, and Parkin-TG mice. B) Representative subcellular fractionation and immunoblot for ubiquitin in nuclear and cytosolic fractions from heart lysates from WT, and Parkin-TG mice. C) Representative subcellular fractionation and immunoblot for endogenous Parkin in the nuclear and cytosolic fractions of WT mouse tissues. Blots are representative of at least n=3 experimental replicates.

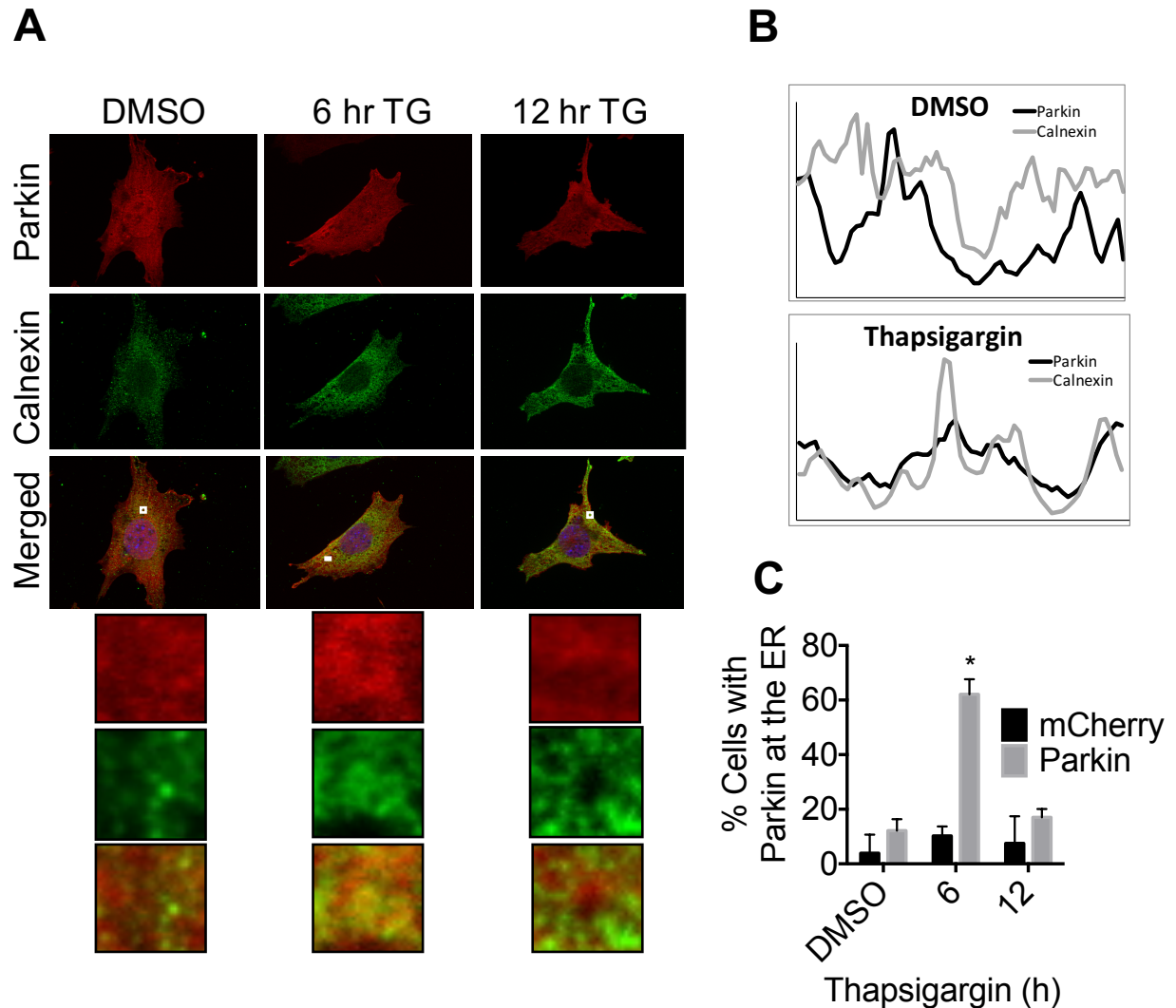


Figure 2.4. Parkin Translocates to the ER in Response to Thapsigargin Treatment.
A) Representative fluorescence microscopy images of HeLa cells expressing mCherry-Parkin and treated with either DMSO or Thapsigargin and fixed at the indicated time points. B) Representative line scans of Parkin co-localization with ER-marker, Calnexin when treated with either DMSO or Thapsigargin for 6 hours. C) Quantification of percent cells with co-localization between either mCherry or Parkin and ER marker, Calnexin. A minimum of 100 cells were counted for n=3 experimental replicates. *p<0.01, compared to DMSO.

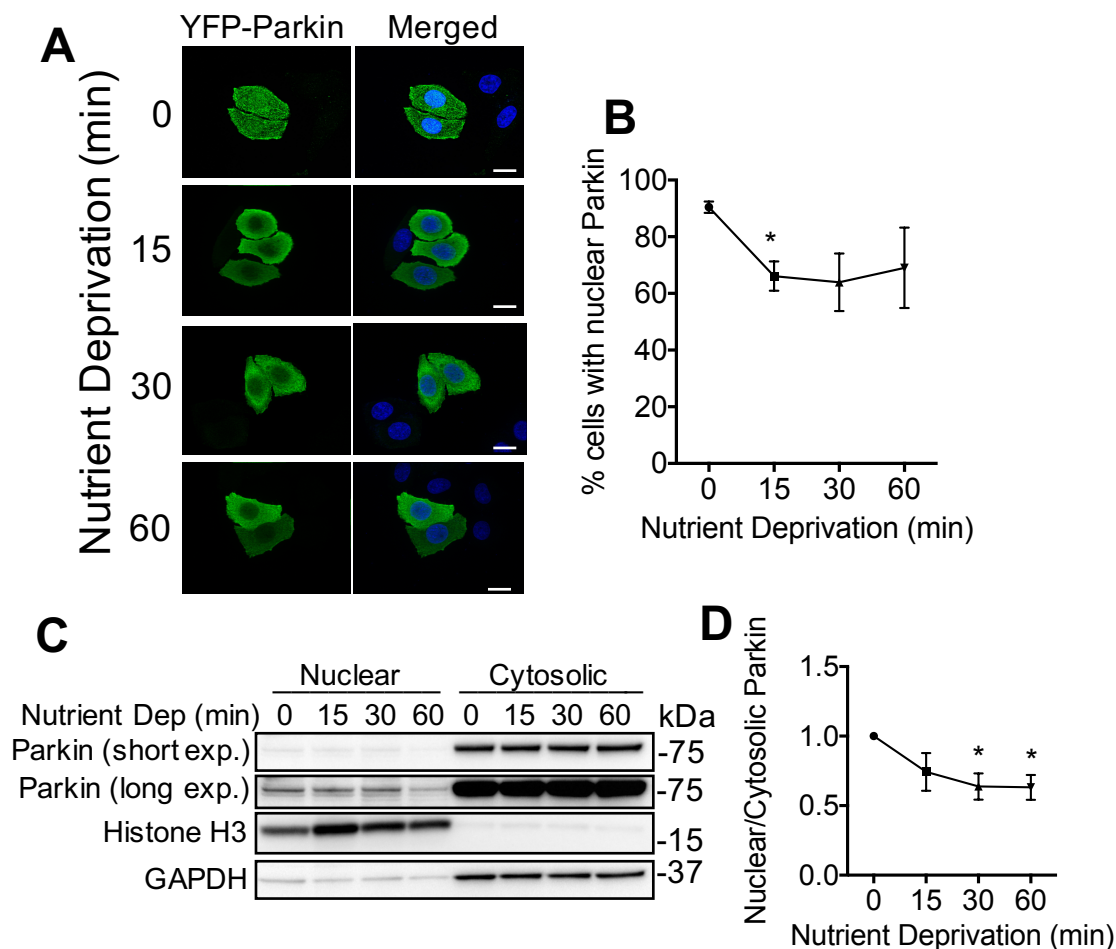


Figure 2.5. Parkin Exits the Nucleus in Response to Nutrient Deprivation. A) Representative fluorescence microscopy images of YFP-Parkin HeLa cells during nutrient deprivation fixed at indicated time points. B) Quantification of presence or absence of Parkin in the nucleus of YFP-Parkin HeLa cells subjected to nutrient deprivation, a minimum of 100 cells counted per condition across three experimental replicates. C) Representative immunoblot of protein levels of Parkin in nuclear and cytosolic fractions of YFP-Parkin HeLa cells during nutrient deprivation. D) Quantification of nuclear Parkin protein levels relative to cytosolic Parkin protein levels in YFP-Parkin HeLa cells subjected to nutrient deprivation, $n=3$, scale bars represent 20 μm , * $p<0.05$ compared to 0 hr.

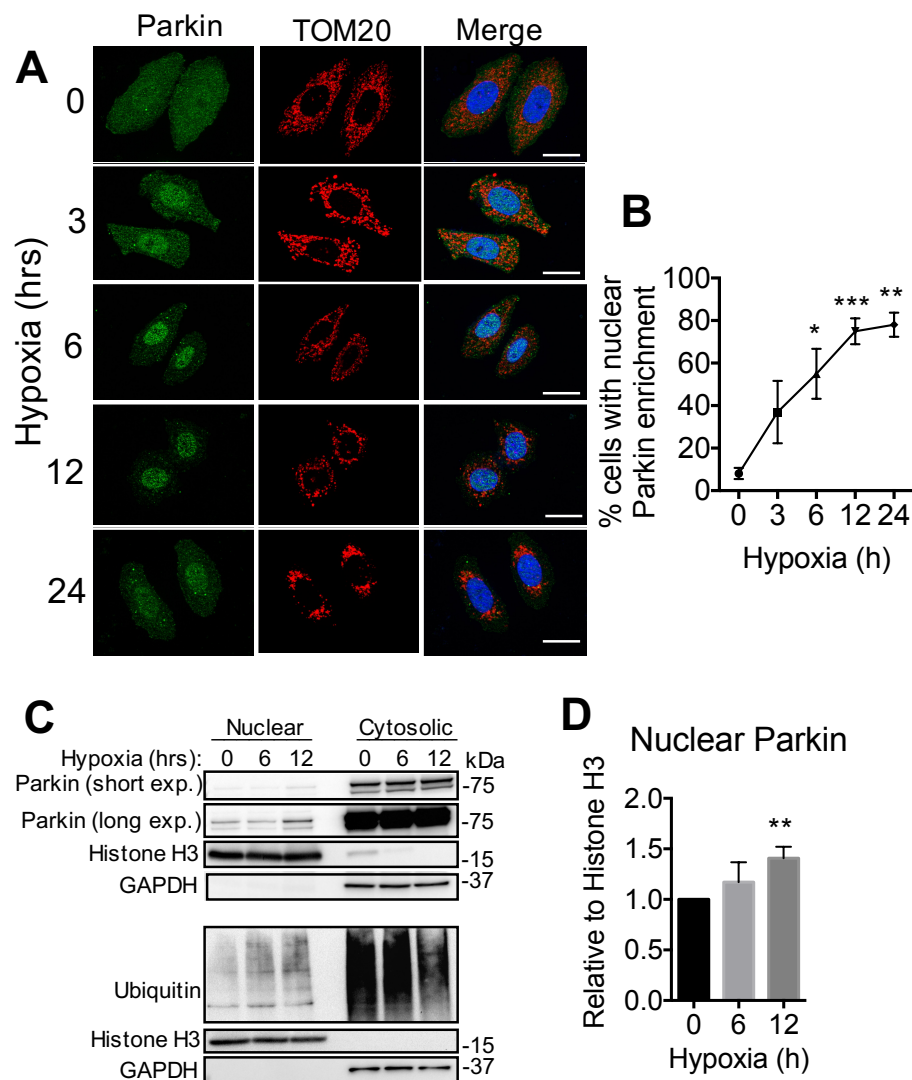


Figure 2.6 Parkin Translocates to the Nucleus During Hypoxia. A) Representative fluorescence microscopy images of YFP-Parkin HeLa cells subjected to hypoxia and fixed at indicated time points. B) Quantification of presence or absence of Parkin enrichment in the nucleus of YFP-Parkin HeLa cells subjected to hypoxia, a minimum of 100 cells counted per condition across three experimental replicates. C) Representative subcellular fractionation and immunoblot of nuclear and cytosolic fractions of HeLa cells expressing mChy-Parkin. D) Quantification of Parkin relative to Histone H3 in the nuclear fraction of HeLa cells expressing mChy-Parkin subjected to hypoxia at indicated time points, n=5. Scale bars represent 20 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0 hr.

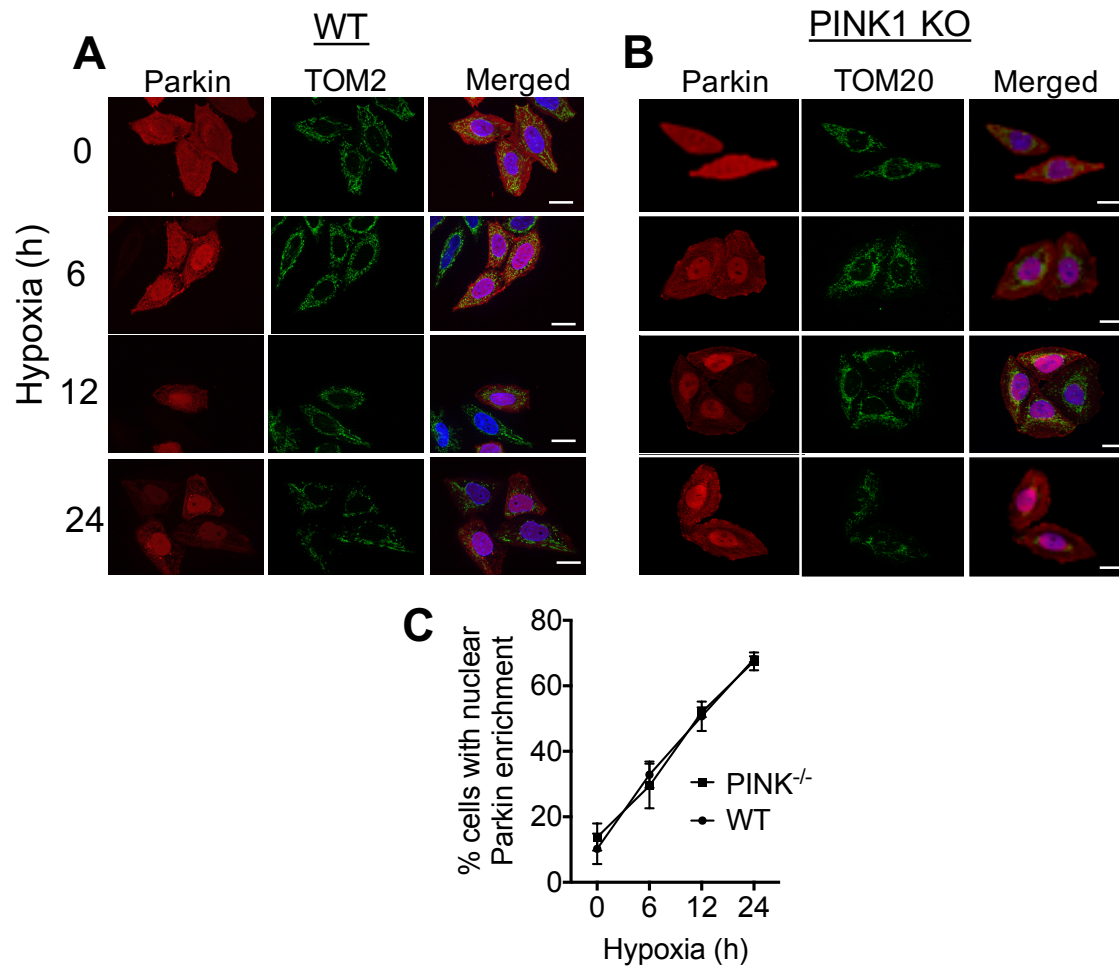
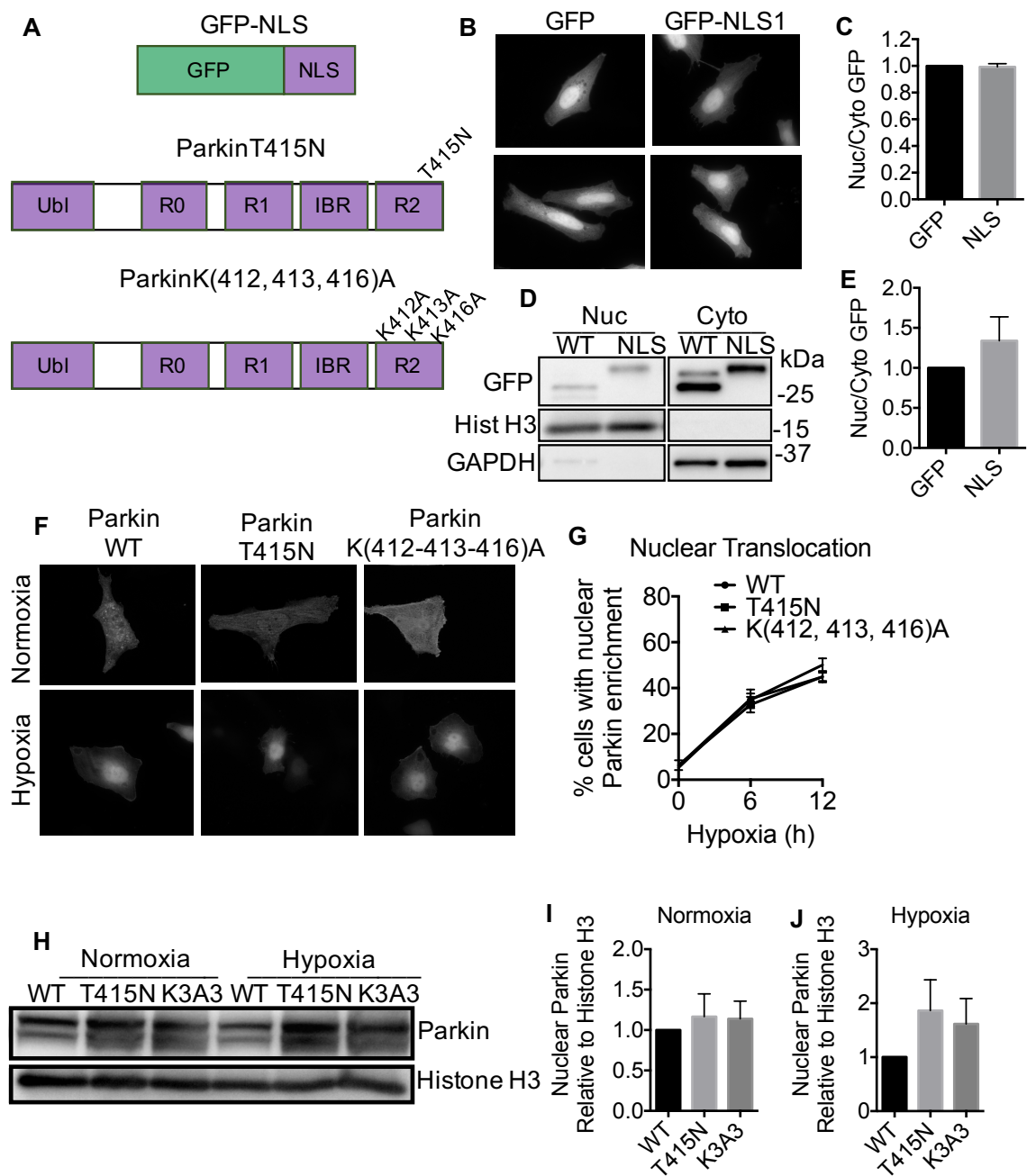


Figure 2.7 Parkin Translocates to the Nucleus Independently of PINK1. A) Representative fluorescence microscopy images of WT HeLa cells expressing mChy-Parkin subjected to hypoxia at indicated time points, images are representative of n=3 experimental replicates. B) Representative fluorescence microscopy images of PINK1^{-/-} HeLa cells expressing mChy-Parkin subjected to hypoxia at indicated time points. Images are representative of n=3 experimental replicates C) Quantification of presence or absence of Parkin enrichment in the nucleus of WT or PINK1^{-/-} HeLa cells subjected to hypoxia, a minimum of 100 cells counted per condition across three experimental replicates. Scale bars represent 20 μ m.

Figure 2.8 Parkin does not have an Endogenous Nuclear Localization Signal. A) Diagram of GFP-NLS, ParkinT415N, and ParkinK(412, 413, 416)A constructs. B) Representative fluorescence microscopy images of HeLa cells expressing GFP or GFP-NLS, images are representative of n=3 experimental replicates. C) Quantification of nuclear vs cytosolic GFP pixel intensity, a minimum of 100 cells counted per condition across three experimental replicates. D) Representative subcellular fractionation and immunoblot of GFP in HeLa cells expressing either GFP or GFP-NLS, immunoblots are representative of n=3 experimental replicates. E) Quantification of GFP from the nuclear vs cytosolic fractions in HeLa cells expressing either GFP or GFP-NLS, n=3. F) Representative fluorescence microscopy images of HeLa cells expressing WT Parkin, ParkinT415N, or Parkin K(412, 413, 416)A, images are representative of n=3 experimental replicates. G) Quantification of nuclear vs cytosolic Parkin (mChy) pixel intensity, a minimum of 100 cells counted per condition across three experimental replicates. H) Representative subcellular fractionation and immunoblot of Parkin in HeLa cells expressing either WT Parkin, ParkinT415N, or ParkinK(412, 413, 416)A at baseline or in response to 12h hypoxia. Immunoblots are representative of n=3 experimental replicates. I and J) Quantification of Parkin from the nuclear vs cytosolic fractions in HeLa cells expressing either WT Parkin, ParkinT415N, or ParkinK(412, 413, 416)A at baseline (I) or in response to 12 h hypoxia (J), n=3.



CHAPTER 3: NUCLEAR PARKIN PROMOTES MITOCHONDRIAL CLEARANCE AND ALTERATIONS IN METABOLIC GENE TRANSCRIPTION

3.1 Introduction

PD is a complex disorder, and the mechanisms that trigger the associated dopaminergic cell death are unclear. Specific signaling pathways, such as mitophagy, protein folding, and proteasomal degradation, are often explored to deconstruct mechanisms for disease pathogenesis. However, in addition to individual signal transduction pathways, more global changes, such as altered metabolic factors, are also linked to the development of neurodegenerative diseases (Agis-Balboa et al., 2011; Shin et al., 2015; Zhou et al., 2003). Recently, several transcriptomic studies using blood, skin biopsies, and pre-frontal cortex brain tissue from patients with PD have found thousands of differentially expressed genes (DEGs) mapping to several pathways including, but not limited to, autophagy, metabolism, MAPK signaling, and immune regulation (Bao et al., 2017; Dumitriu et al., 2016; Grozdanov et al., 2014; Miki et al., 2018; Papagiannakis et al., 2015; Planken et al., 2017; Shamir et al., 2017). Although the primary aims of these studies were discovering additional environmental or genetic contributors and characterizing markers for early diagnostics, they may also be useful in understanding disease mechanisms. Additionally, the functional contribution of nuclear-localized Parkin to the transcriptome is unknown.

I have shown that Parkin localizes to the nucleus at baseline, and its nuclear localization is dynamic during various cellular stress conditions (Chapter 2). In this chapter, I assess the consequences of Parkin in the nucleus. To separate Parkin's

functions in different cellular compartments, I generate targeted constructs that sequester Parkin to either the mitochondria or the nucleus. Using immunofluorescence and western blotting, I establish whether nuclear Parkin plays a role in mitochondrial clearance. I also use RNA sequencing to investigate transcriptomic changes in response to nuclear Parkin in HeLa cells at baseline and in response to hypoxia.

3.2 Results

3.2.1 Generation of Targeted Parkin Constructs

I have shown in Chapter 2 that Parkin localizes throughout the cell under baseline conditions. Therefore, to separate the functions of Parkin in each cellular compartment, we generated nuclear- and mitochondrial-targeted Parkin constructs, NLS-Parkin and Mito-Parkin, respectively. To generate the nuclear-targeted Parkin construct, I inserted three consecutive nuclear localization signals (NLS) between mCherry and Parkin. Mito-Parkin was created by fusing the N-terminal 33 amino acids of outer mitochondrial membrane protein, Translocate of the Outer Mitochondrial Membrane 20 (TOM20) to the 5'-end of mCherry-Parkin (Figure 3.1 A). While Parkin localizes throughout the cell under baseline conditions when overexpressed in MEFs, Mito-Parkin localizes to the mitochondria, and NLS-Parkin is restricted to the nucleus (Figure 3.1 B). Treating cells with the mitochondrial uncouplers, such as FCCP, induces Parkin to translocate to the mitochondria (Narendra et al., 2008). To confirm that NLS-Parkin remains in the nucleus, I exposed HeLa cells and MEFs overexpressing Parkin or NLS-Parkin to FCCP. While WT Parkin translocates to the mitochondria following FCCP treatment, NLS-Parkin remains in the nucleus (Figure 3.2). These data indicate

that the targeted constructs we generated localize to their respective subcellular compartments.

3.2.2 Nuclear Parkin Contributes to Mitochondrial Clearance in Response to Mitochondrial Uncouplers

Parkin rapidly translocates to dysfunctional mitochondria where it ubiquitinates proteins in the outer mitochondrial membrane. These ubiquitinated proteins serve to mark the mitochondria for autophagic degradation. It is unknown whether nuclear Parkin also contributes to mitochondrial clearance. Therefore, I examined the ability of Parkin to facilitate mitochondrial clearance from different cellular compartments in response to various mitochondrial stressors. First, I assessed the ability of NLS-Parkin and Mito-Parkin's to clear mitochondria in response to FCCP treatment. To measure mitochondrial clearance, I expressed either mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin in MEFs, exposed them to FCCP for up to 24 hours, and counted the percentage of cells undergoing mitochondrial clearance (Figure 3.3). Although cells cleared their mitochondria in the presence of all three Parkin constructs, cells expressing Mito-Parkin cleared their mitochondria at an accelerated rate compared to WT Parkin, while cells expressing NLS-Parkin cleared their mitochondria at a slower rate and to a lesser extent than WT Parkin. In line with previous observations (Hammerling et al., 2017), cells expressing mCherry alone did not clear their mitochondria in response to FCCP treatment.

I next confirmed that NLS-Parkin-facilitated mitochondrial clearance was not limited to FCCP treatment. While FCCP depolarizes mitochondria by increasing their

permeability to hydrogen ions, Valinomycin causes depolarization by increasing mitochondrial membrane permeability to potassium ions. Additionally, treating cells with Oligomycin and Antimycin A uncouples mitochondria by disrupting electron transport chain components ATP synthase and cytochrome c reductase, respectively. To establish whether NLS-Parkin induces mitochondrial clearance in response to treatment with these additional mitochondrial uncoupling agents, we exposed MEFs expressing mCherry, WT Parkin, NLS-Parkin, or Mito-Parkin to Valinomycin or Oligomycin/Antimycin A (Figure 3.4). Similar to FCCP treatment, cells expressing mCherry did not clear their mitochondria, while cells expressing either WT Parkin, Mito-Parkin, or NLS-Parkin did in response to both Valinomycin (Figure 3.4 A) and Oligomycin/Antimycin A treatment (Figure 3.4 B). Additionally, cells expressing NLS-Parkin cleared their mitochondria to a lesser extent than WT and Mito-Parkin. Taken together, these data suggest that NLS-Parkin contributes to mitochondrial clearance in response to mitochondrial depolarization.

Because NLS-Parkin remains in the nucleus instead of translocating to damaged mitochondria during FCCP treatment, I sought to determine the mechanism of mitochondrial clearance. In addition to the PINK1/Parkin pathway, mitophagy can be facilitated via receptors that reside on the outer mitochondrial membrane, such as BNIP3, NIX, and FUNDC1. Mitophagy receptors bind directly to LC3II on the autophagosome and do not require ubiquitination to promote mitophagy. Therefore, I interrogated whether nuclear Parkin increases transcription of mitophagy receptors. To measure transcription of mitophagy receptors in response to nuclear Parkin, I expressed either mCherry, WT Parkin, or NLS-Parkin in HeLa cells and performed a

qPCR for transcript levels of BNIP3, NIX, and FUNDC1 (Figure 3.5). Although cells expressing mCherry and WT Parkin had similar levels of all three transcripts, cells expressing NLS-Parkin had increased transcript levels of FUNDC1. Collectively, these data suggest that nuclear Parkin contributes to mitochondrial-stress-induced mitophagy, possibly through increasing the transcription of mitophagy receptor FUNDC1.

3.2.3 Nuclear Parkin does not Facilitate Mitochondrial Clearance in Response to Hypoxia

In Chapter 2, I found that Parkin translocates to the nucleus in response to hypoxia. My previous data in this chapter indicate that nuclear-targeted Parkin contributes to mitochondrial clearance in response to uncoupling agents. Therefore, I addressed whether nuclear Parkin facilitates mitochondrial clearance in response to hypoxia. To measure mitochondrial clearance, I expressed either mCherry, WT Parkin, or NLS-Parkin in HeLa cells and subjected them to either normoxia or hypoxia for 24 hours and quantified relative protein levels of several OXPHOS proteins (Figure 3.6 A and B). Although I did measure a slight decrease in protein levels at baseline in some of the OXPHOS proteins in response to NLS-Parkin expression, there was no detectable effect of Parkin expression—either WT or NLS—in response to hypoxia. I confirmed my previous results by quantifying mitochondrial DNA (mtDNA) content via qPCR (Figure 3.6 C). Collectively, these data suggest that nuclear Parkin does not mediate mitochondrial clearance in response to hypoxia.

3.2.4 Effect of nuclear Parkin on the transcriptome

To further understand the effects of nuclear Parkin, we performed next-generation RNA sequencing (RNA-seq) on HeLa cells overexpressing mCherry, Parkin, or NLS-Parkin under baseline and hypoxic conditions. Hierarchical clustering of differentially expressed genes (DEGs; fold-change ≥ 1.5 , FDR < 0.05) revealed no effect for WT Parkin on the basal transcriptome; however, NLS-Parkin significantly altered the expression of 168 genes during normoxic conditions (Figure 3.7 A). Of the differentially expressed genes (DEGs), 116 (69%) were downregulated while 52 (31%) were induced as a result of NLS-Parkin. The grouping of samples and directionality of expression patterns are further visualized in a hierarchical clustering heatmap of all DEGs (Figure 3.7 B). Next, we employed EnrichR (Kuleshov et al., 2016) to evaluate the biological pathways implicated by NLS-Parkin-mediated DEGs. Using 3 different human pathway analysis tools within the EnrichR platform, the 5 most significantly enriched pathways associated with the NLS-Parkin transcriptome were ranked. Several pathways reflected amino acid and ketone metabolism, which suggests a potential bioenergetic role for NLS-Parkin-mediated transcription (Figure 3.7 C). Indeed, we noted multiple nuclear receptors (PPARG, PPARA, ESR1) and co-activators (NCOA1) as potential upstream regulators of a subset of metabolic transcripts among the NLS-Parkin DEGs (Figure 3.8). Taken together, these data demonstrate that targeting Parkin to the nucleus affects transcription of genes involved in multiple metabolic pathways.

Because Parkin dynamically translocates to the nucleus during hypoxia, we subjected HeLa cells expressing either mCherry, Parkin, or NLS-Parkin to hypoxia and re-evaluated global transcriptional changes by RNA-seq. Multi-Dimensional Scaling (MDS) revealed a marked difference in the transcriptomes of normoxic and hypoxic

samples used in our study. However, in contrast to the clustering of the WT Parkin and mCherry replicates observed at normoxia, the transcriptome of WT Parkin-expressing cells shifted towards NLS-Parkin indicating a dose wise effect for nuclear Parkin on hypoxic gene expression (Figure 3.9 A). Along these lines, despite no role for WT Parkin on basal gene expression (see Figure 3.7 A), WT Parkin during hypoxia was sufficient to alter the expression of 158 genes (fold-change ≥ 1.5 , FDR < 0.05), of which 37% were shared with the NLS-Parkin transcriptome (Figure 3.9 B). Consistent with a dose-dependent effect, NLS-Parkin significantly altered the abundance of 585 transcripts during hypoxia (Figure 3.9 B). To exclude indirect transcriptional changes and identify the potential direct downstream effects of Parkin in the nucleus, we focused our attention on the overlapping DEGs shared by WT and NLS Parkin during hypoxia. Importantly, all 58 DEGs within this subset were up (33%) or downregulated (67%) similarly in WT and NLS Parkin cells highlighting the specificity of this pool of Parkin-dependent transcripts (Figure 3.9 B). Hierarchical clustering of these hypoxia-responsive shared DEGs demonstrated similar magnitudes of change among WT and NLS Parkin replicates (Figure 3.10 A). qPCR validated two genes with increased and two with decreased transcript abundance (Figure 3.10 B). Pathway analysis revealed significant enrichment of HIF-1 signaling, the heat shock protein response, and various metabolic processes (Figure 3.10 C). Using the chromatin immunoprecipitation enrichment (ChEA) tool within EnrichR, we sought to examine potential upstream regulators of the overlapping DEG subset shared by WT and NLS Parkin. Similar to our analysis of NLS-mediated DEGs during normoxia (see Figure 3.8), several nuclear receptors known to regulate metabolic gene expression emerged as potential transcriptional regulators of

the shared hypoxia-responsive DEGs among WT and NLS-Parkin (Figure 3.11). Collectively, these data demonstrate a significant role for Parkin in regulating hypoxic gene expression, and suggest that downstream induction of metabolic transcripts may occur through interaction with one or more nuclear receptors.

3.3 Discussion

The present study demonstrates several effects of Parkin in the nucleus. First, I show that nuclear Parkin promotes mitochondrial clearance in response to mitochondrial uncoupling agents. Uncouplers are generally used to stimulate Parkin translocation to the mitochondria. As NLS-Parkin remains in the nucleus, understanding how Parkin facilitates clearance is a topic of interest. My data show that cells expressing NLS-Parkin have increased transcript levels of FUNDC1, suggesting nuclear Parkin may promote mitophagy through its upregulation. Future studies will examine mechanisms of nuclear-Parkin-mediated mitochondrial clearance to parse out which downstream pathways are involved.

In response to mitochondrial depolarization, Parkin robustly translocates to the mitochondria. Whether Parkin also translocates to, or is active in, other cellular compartments under these conditions is unclear. It is possible that a portion of Parkin translocates to the nucleus in addition to the mitochondria. Additionally, if Parkin translocation to the mitochondria fails, nuclear Parkin may be able to compensate for partial lack of mitochondrial Parkin. Additional studies are required to understand the physiological context for nuclear Parkin during mitochondrial depolarization.

Interestingly, in the context of hypoxia, where I had previously measured Parkin translocation to the nucleus, I do not see Parkin-mediated mitochondrial clearance, indicating that Parkin function is cellular context dependent. Therefore, I used RNA-seq to determine the role of nuclear Parkin both at baseline and in response to hypoxia. Overall, the RNA-seq study showed that Parkin does not play a role in gene expression under basal conditions; but that during hypoxia, it functions to regulate transcription of genes involved in metabolism. Once again, these data indicate that Parkin's role in the cell—and in different cellular compartments—is highly context-dependent. Although there have been other studies that evaluated Parkin's role in gene transcription, this study reports the function of Parkin specifically when localized to the nucleus, and in the physiologic context of hypoxia.

3.4 Materials and Methods

Cell Culture

Cells were plated and maintained as described previously (Hammerling et al., 2017). MEFs and HeLa cells were plated and maintained in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher) containing 10% fetal bovine serum (FBS, ThermoFisher) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), and cultured in 5% CO₂ atmosphere at 37°C. Hypoxia was carried out via replacing culture media with media supplemented with 20mM HEPES pH 7.4 (ThermoFisher), and placing culture dishes into BD GasPak EZ pouch (BD Biosciences) filled with nitrogen and sealed or back in the 5% CO₂ atmosphere incubators for normoxic controls.

Adenoviral Constructs

We generated the mCherry-Parkin virus from the plasmid (Addgene #23956). Mito-Parkin, was created by fusing the N-terminal 33 amino acids of TOM20 to the 5'-end of mCherry-Parkin. The partial-TOM20 DNA was given to us by Dr. Alexandra Newton (Gallegos et al., 2006). NLS-Parkin was generated by fusing mCherry linked to three consecutive SV40 nuclear localization signals (pmCherry-NLS, Addgene 39319) to the N-terminal end of Parkin. Cells were transiently transfected with DNA using Eugene 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Cells were infected with adenoviruses in DMEM + 2% heat-inactivated serum and rescued with their respective culture media. Treatments and experiments were performed 24 hrs post-infection.

RNA-sequencing

Cells were harvested and total RNA isolated via RNeasy Mini Kit (Qiagen). RNA-sequencing was conducted via Illumina HiSeq2000. Quality control of the raw fastq files was performed using the software tool FastQC v0.11.3. Sequencing reads were trimmed with Trimmomatic v0.36 (Bolger et al., 2014) and aligned to the human genome (GRCh37.p13) using the STAR aligner v2.5.3a (Dobin et al., 2013). Read quantification was performed with RSEM v 1.3.0 (Li and Dewey, 2011) and the Gencode release 19 (Frankish et al., 2019). The R BioConductor packages edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) were used to implement the limma-voom method (Law et al., 2014) for differential expression analysis. In brief, lowly expressed genes—those not having counts per million (cpm) ≥ 1 in at least 4 of the

samples—were filtered out, and then trimmed mean of M-values (TMM) (Robinson and Oshlack, 2010) normalization was applied. The values weighted for inter-subject correlations in repeated measures of samples, after which lmFit was used to fit per-gene linear models and empirical Bayes moderation was applied with the eBayes function. Significance was defined by using an adjusted p-value cut-off of 0.05 after multiple testing correction (Benjamini et al., 2001) using a moderated t-statistic in limma and a minimum absolute log-fold-change threshold of 1.5.

Quantitative PCR

RNA extraction, genomic DNA extraction, cDNA synthesis, and quantitative PCR were performed as described previously (Orogo et al., 2015). Briefly, genomic DNA and total RNA were isolated from cells using the GenElute Mammalian Genomic DNA Miniprep Kit (MilliporeSigma) and the RNeasy Mini Kit (Qiagen), respectively, following the manufacturers protocols. cDNA was synthesized according to the manufacturer's protocol using the QuantiTect Reverse Transcription kit (Qiagen). TaqMan primers for *Bnip3*, *Bnip3l/NIX*, *Fundc1*, *mT_7s* (mtDNA), *ASNS*, *HKDC1*, *DYNLT3*, *MYLK3*, and *Rn18s* and TaqMan Universal Master Mix II was purchased from Applied Biosystems/Life Technologies. Primer sequences are available in Table S1. qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Relative mRNA was normalized to *Rn18s*, and fold change in gene expression was calculated using the $2^{(-\Delta\Delta C_t)}$ method.

Fluorescence Microscopy

Cells were fixed, permeabilized, and blocked as described previously (Hammerling et al., 2017). The cells were stained with anti-TOMM20 (Santa Cruz Biotechnology, 11415) to label mitochondria and incubated with Alexa Fluor 488 or 594 secondary antibodies (Life Technologies A11037) and Hoechst 33342 (Life Technologies, H3570) to stain nuclei. Cells were imaged with a Carl Zeiss AxioObserver Z1 fitted with a motorized Z-stage. Z-stacks were acquired in ApoTome mode using a high-resolution AxioCam MRm digital camera, a 63X Oil-immersion objective and Zeiss AxioVision 4.8 software (Carl Zeiss).

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Graphed data were represented as mean $\pm$ SEM. Unpaired Student's t test was used for two-group comparisons. One- or two-way ANOVA with post hoc comparisons by Tukey's method, paired, or unpaired t test was used for the comparisons between multiple groups, as appropriate.

Parts of Chapter 3 were submitted for publishing. Shires, S. E., Quiles, J. M., Najor, R. H., Leon, L. J., Cortez, M. Q., Lampert, M. A., Mark, A., Gustafsson, Å. B. Nuclear Parkin Promotes the ER α -mediated Transcriptional Program and Drives Widespread Changes in Gene Expression Following Hypoxia. The dissertation author was the primary investigator and author of this paper.

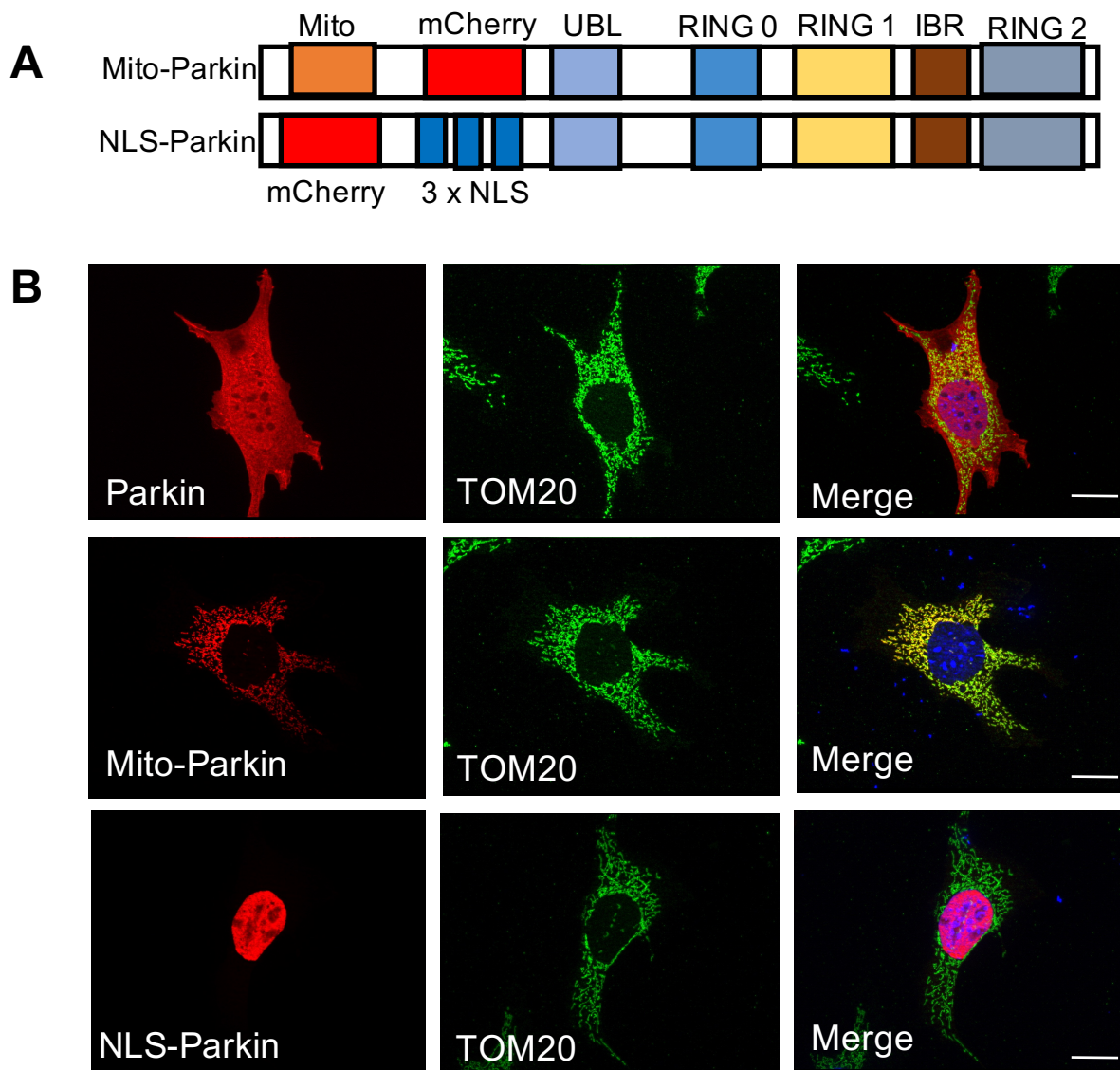


Figure 3.1 Generation of Targeted Parkin Constructs A) Diagram of mitochondrial- and nuclear-targeted Parkin constructs. B) Representative fluorescence microscopy images of MEFs expressing WT Parkin, Mito-Parkin, or NLS-Parkin. Scale bars represent 20 μm .

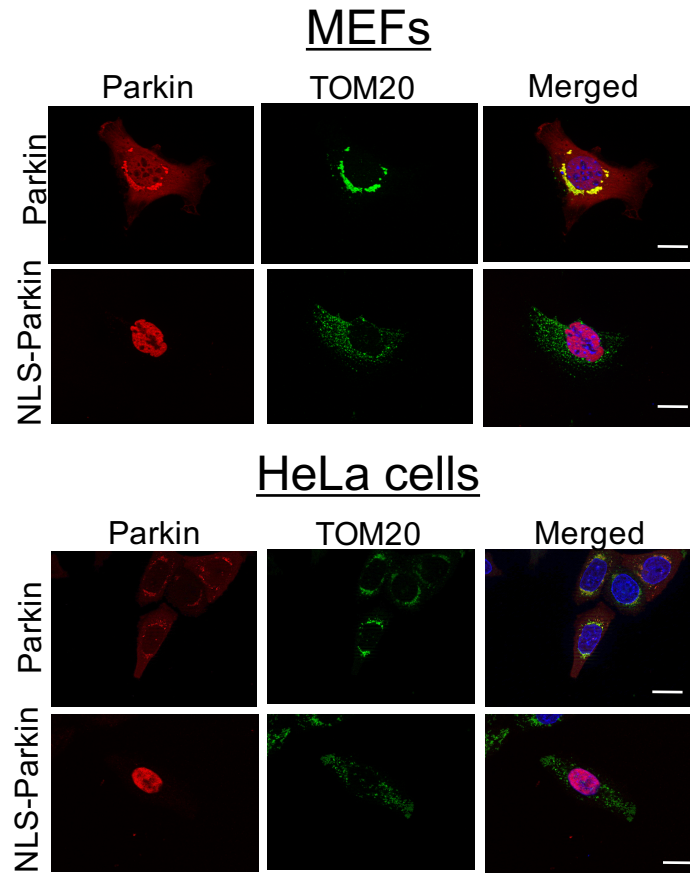


Figure 3.2 NLS-Parkin Remains in the Nucleus in Response to FCCP. Representative fluorescence microscopy images of MEFs (top) and HeLa cells (bottom) expressing WT Parkin, Mito-Parkin, or NLS-Parkin after treatment with 25 μ M FCCP for 12 hours. Scale bars represent 20 μ m

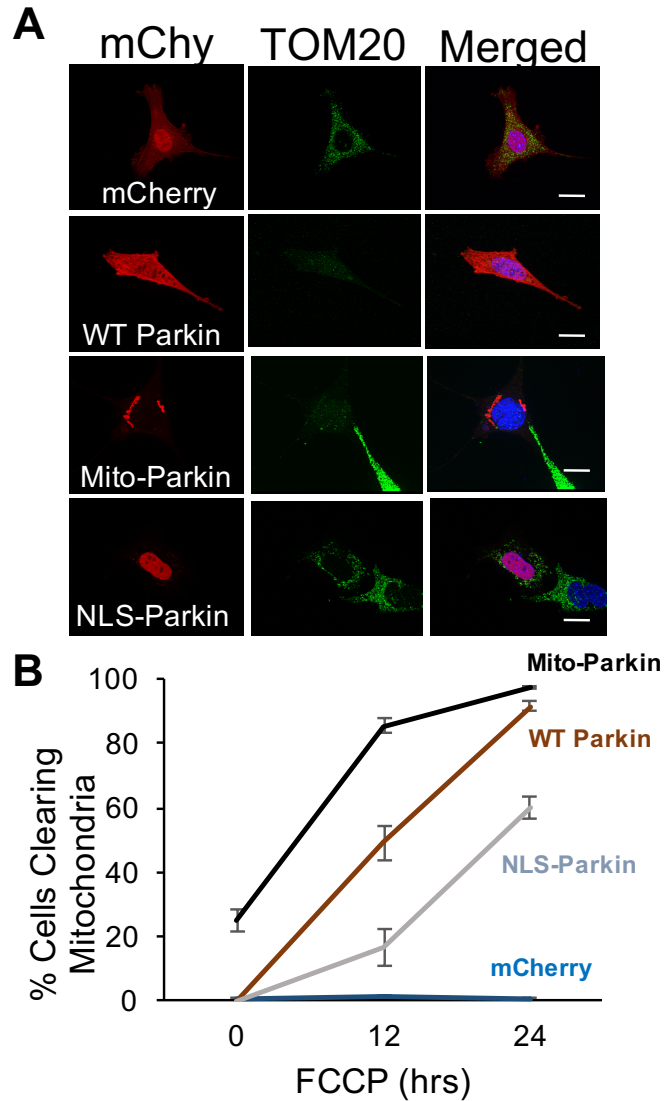


Figure 3.3 Nuclear Parkin Contributes to Mitochondrial Clearance in Response to FCCP. A) Representative fluorescence microscopy images of MEFs expressing mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin after treatment with 25 μ M FCCP for 12 hours. Scale bars represent 20 μ m. B) Quantification of percent of cells undergoing mitochondrial clearance in MEFs expressing either mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin and treated with either DMSO or FCCP for 12 or 24 hours, n=3.

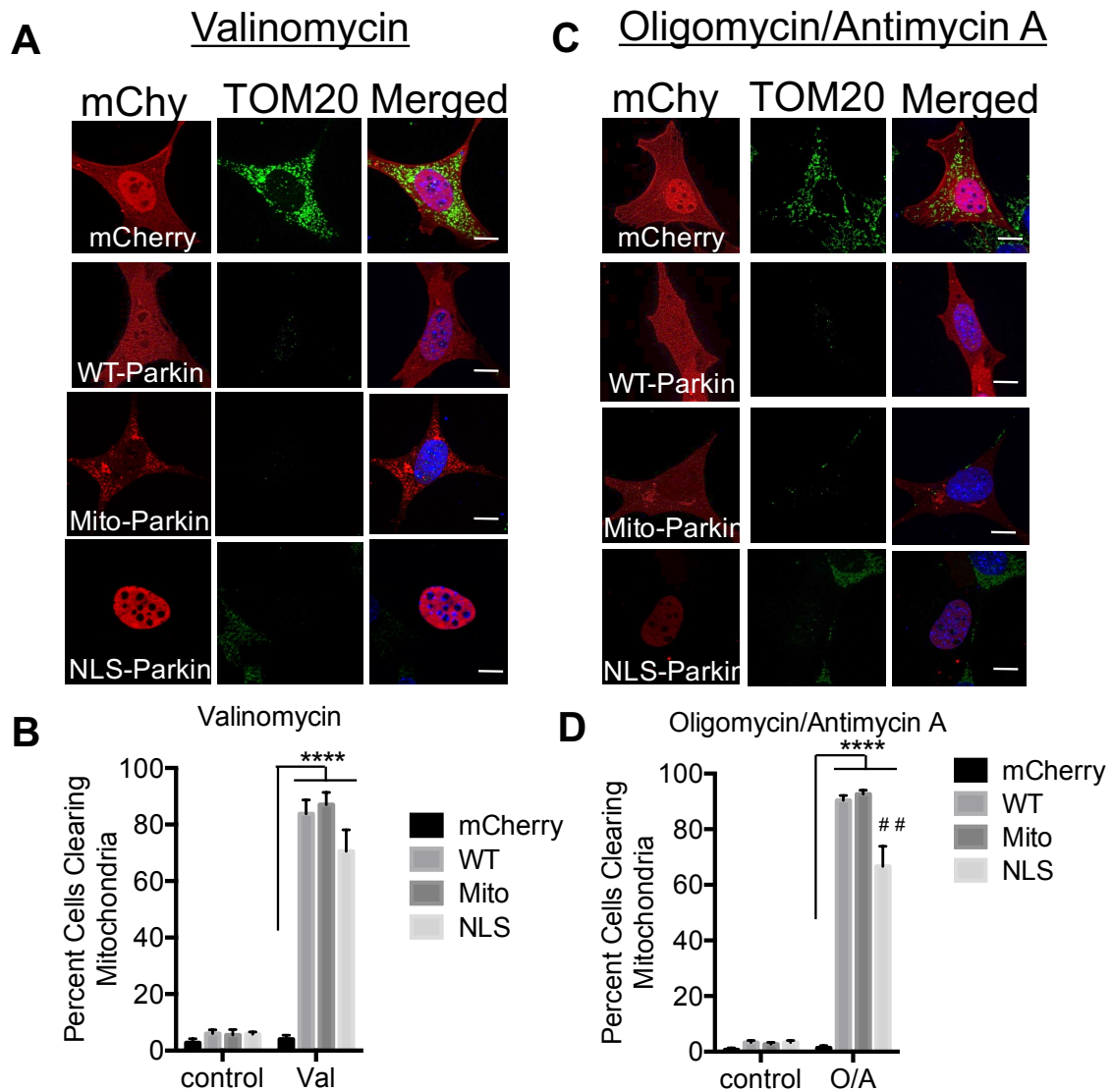


Figure 3.4 Nuclear Parkin Contributes to Mitochondrial Clearance in Response to Valinomycin and Oligomycin/Antimycin A. A) Representative fluorescence microscopy images of MEFs expressing mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin after treatment with 10 μ M Valinomycin (Val) for 24 hours. B) Quantification of percent of cells undergoing mitochondrial clearance in MEFs expressing either mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin and treated with either vehicle or Valinomycin for 24 hours, $n=4$, **** $p<0.0001$ compared to control treatment. C) Representative fluorescence microscopy images of MEFs expressing mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin after treatment with 2.5 μ M Oligomycin/ 10 μ M Antimycin A (O/A) for 24 hours. D) Quantification of percent of cells undergoing mitochondrial clearance in MEFs expressing either mCherry, WT Parkin, Mito-Parkin, or NLS-Parkin and treated with either vehicle or Oligomycin/Antimycin A for 24 hours, $n=3$, **** $p<0.0001$ compared to control treatment. ## $p<0.01$ compared to WT Parkin.

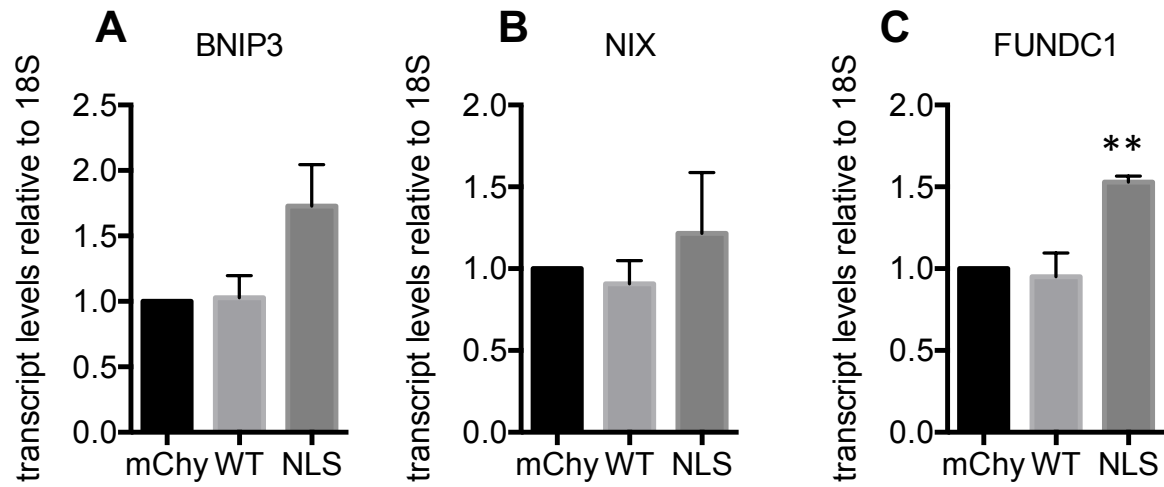


Figure 3.5 Cells Expressing NLS-Parkin have Increased Transcript Levels of FUNDC1. Transcript levels of mitophagy receptors BNIP3 (A), NIX (B), and FUNDC1 (C) in HeLa cells expressing either mCherry, WT Parkin, or NLS-Parkin, n=3, **p<0.01 compared to mCherry.

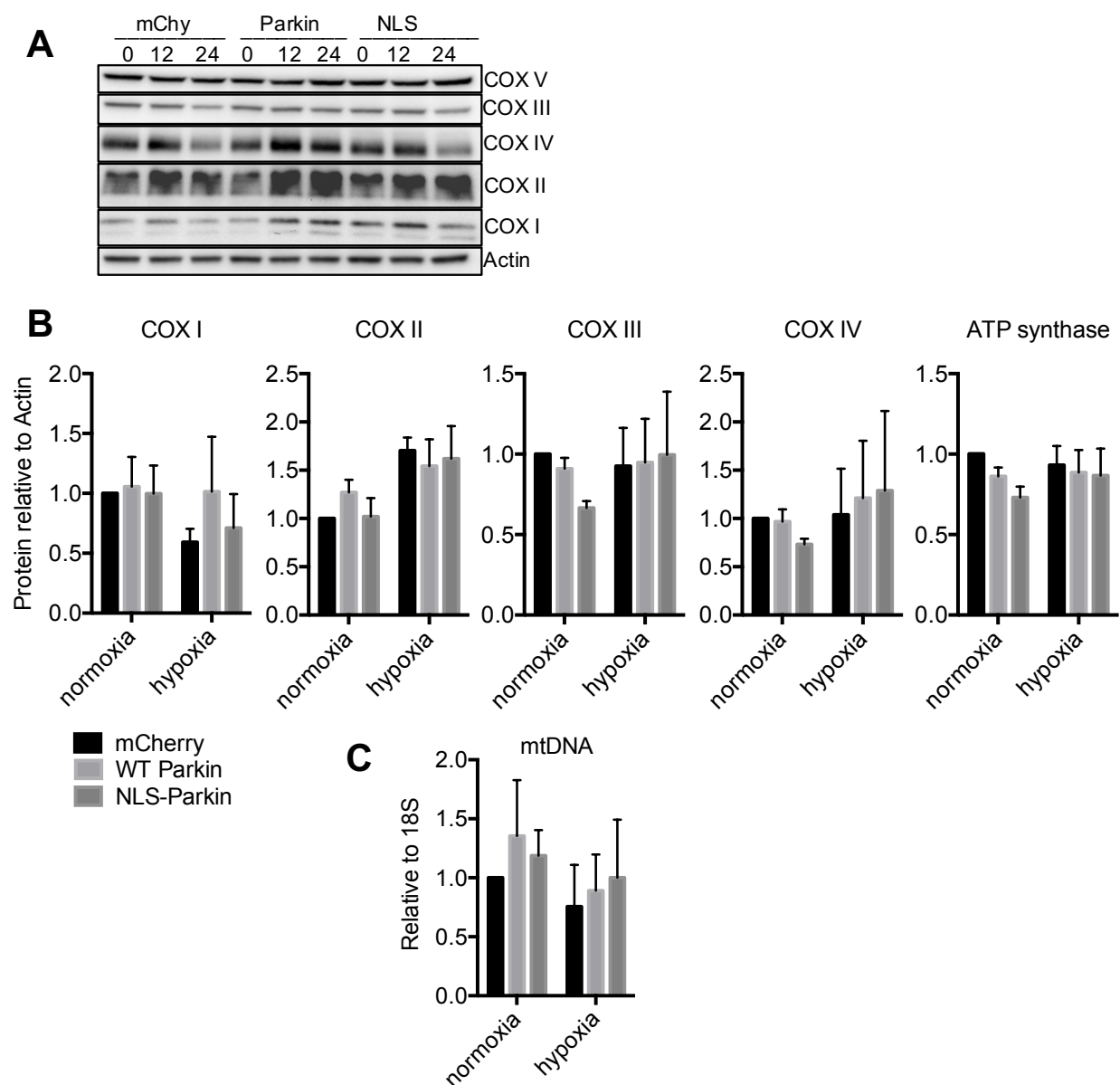


Figure 3.6 Nuclear Parkin does not Facilitate Mitochondrial Clearance in Response to Hypoxia. A) Representative immunoblot of protein levels of OXPHOS proteins in HeLa cells expressing mCherry, WT Parkin, or NLS-Parkin at baseline and in response to 12 or 24 hours of hypoxia. Immunoblot is representative of n=5. B) Quantification of OXPHOS protein levels relative to Actin in HeLa cells expressing mCherry, WT Parkin, or NLS-Parkin at baseline and in response to 24 hours of hypoxia. n=5. C) Levels of mtDNA relative to genomic DNA in HeLa cells expressing either mCherry, WT Parkin, or NLS-Parkin at baseline and in response to 24 hours of hypoxia. n=3.

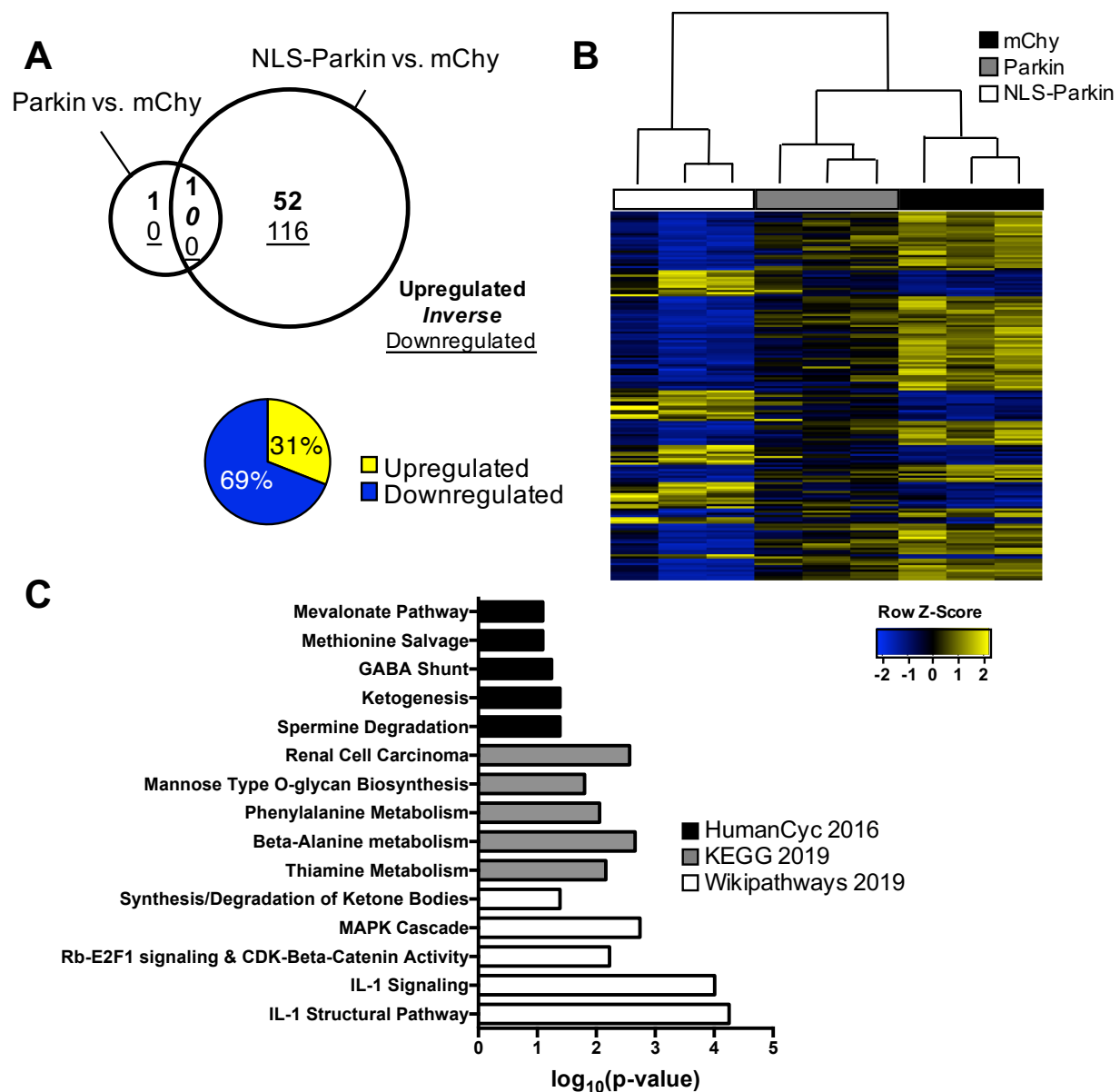


Figure 3.7 Effect of Nuclear Parkin on the Transcriptome at Baseline. A) Area-proportional Venn diagram of the number of differentially expressed genes (DEGs) identified through RNA sequencing (RNA seq) comparing HeLa cells expressing WT Parkin or NLS-Parkin to mCherry-expressing cells. Number of upregulated genes in bold, number of downregulated genes underlined, with percentages of upregulated and downregulated genes depicted in pie chart, n=3. B) Hierarchical clustering heat map of all DEGs, n=3. C) Top five biological processes determined by Gene Ontology analysis of all DEGs from “a” using HumanCyc 2016 (top), KEGG 2019 (middle) and Wikipathways 2019 (bottom), n=3.

Regulator	P-value	Combined score	Regulator	P-value	Combined score	Regulator	P-value	Combined score
IRF8	0.00005635	51.64	NCOA1	1.51E-11	159.74	PPARA	0.02717	9.5
IRF1	0.0001667	33.02	SPDEF	1.64E-11	168.96	HINFP	0.03115	4.74
SOX2	0.0003034	14.29	PBX3	4.36E-10	115.14	SNAI2	0.03122	5.49
NUCKS1	0.0004571	21.8	NCOA1	1.05E-08	96.75	SNAI1	0.03122	5.49
SMAD3	0.00237	12.7	KLF9	1.22E-08	95.02	TCF3	0.03122	5.49
TP63	0.004579	48.09	STAT1	1.25E-08	117.73	ZNF281	0.03853	5.5
SETDB1	0.005519	8.58	IRF1	2.60E-08	92.28	SND1	0.04079	4.91
TFAP2C	0.006005	9.62	DUX4	2.90E-08	105.41	TFAP2C	0.04367	4.77
FOXP2	0.006224	10.51	REST	6.22E-08	88.28			
DNAJC2	0.008904	9.38	KMT2A	1.92E-07	87.98			
TCF21	0.009041	7.56	ZEB1	5.17E-07	58.75			
FOXA1	0.009041	7.56	KMT2A	5.78E-07	81.13			
SCL	0.01988	11.11	HSF1	9.95E-07	68.55			
MYB	0.02386	6.75	POU1F1	0.00000127	65.97			
NANOG	0.02513	6.79	CTCF	0.000001598	75.36			
TCF7L2	0.02568	6.39	FOSL1	0.000001717	68.03			
PPARG	0.02756	5.77	FOSL1	0.000001847	67.21			
MAF	0.02824	5.31	IRF9	0.000002648	76.44			
KLF6	0.02824	5.31	POU5F1	0.00000557	74.62			
KDM2B	0.02824	5.31	HSF1	0.000007417	63.13			
MYC	0.02824	5.31	SOX2	0.00000932	60.43			
ESR1	0.04362	8.18	TWIST1	0.00000948	54.89			
ATF3	0.04368	4.43	LIN28	0.00001072	87.19			
STAT3	0.04478	4.55	ZNF207	0.00001156	48.87			
JUN	0.04697	4.37	TP63	0.00001378	62.26			

Figure 3.8 Potential Upstream Regulators of Nuclear-Parkin Responsive Genes at Baseline. EnrichR output from ChIP Enrichment Analysis (ChEA) (left), TF Perturbations Followed by Expression (middle) and TRANSFAC and JASPAR Position Weighted Matrices (PWMs) (right) showing the top 25 most significantly enriched transcriptional regulators associated with the differentially expressed genes (DEGs) from NLS Parkin vs. mCherry under baseline conditions, n=3.

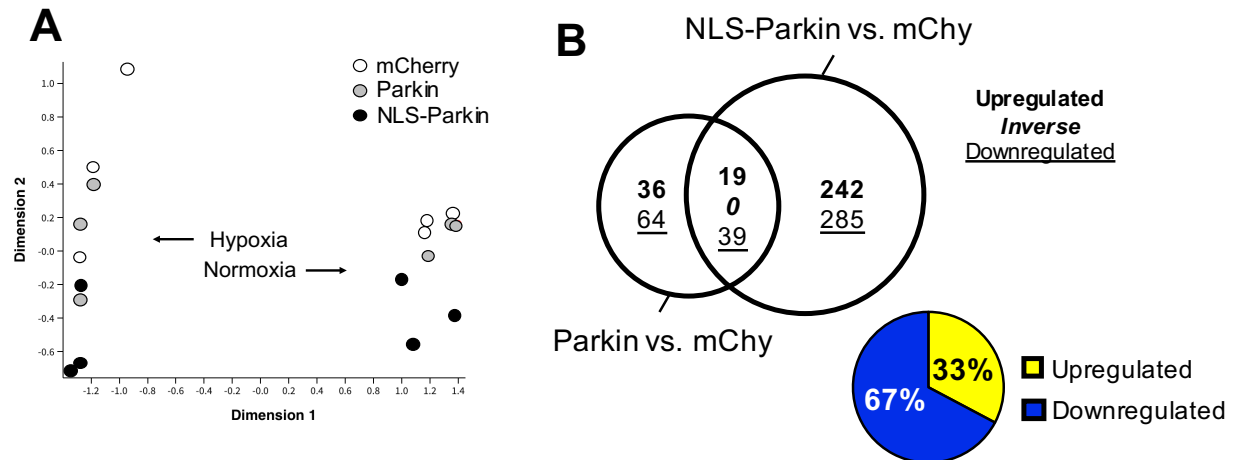


Figure 3.9 Effect of Nuclear Parkin on the Transcriptome During Hypoxia. Multi-dimensional scale (MDS) plot of all of the samples analyzed through RNA sequencing: HeLa cells expressing either mCherry, WT Parkin, or NLS-Parkin at baseline/ normoxia or subjected to 12 hours of hypoxia b) Area-proportional Venn diagram of the number of genes differentially expressed in response to 12 hours of hypoxia, comparing HeLa cells expressing WT Parkin or NLS-Parkin to mCherry-expressing cells. Number of upregulated genes in bold, number of downregulated genes underlined, with percentages of upregulated and downregulated genes depicted in pie chart (right), n=3.

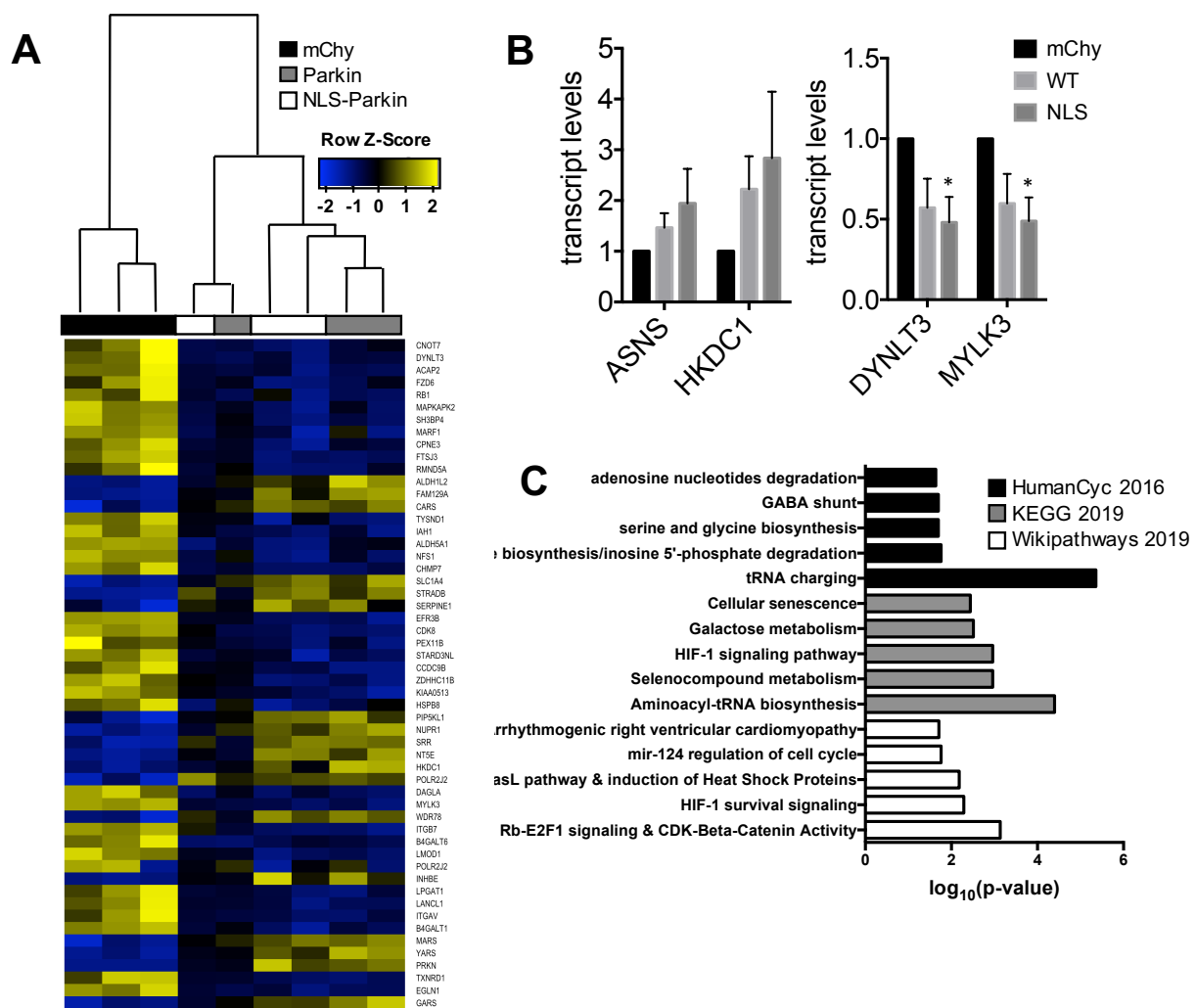


Figure 3.10 Nuclear Parkin-Responsive Differentially Expressed Genes During Hypoxia. A) Hierarchical clustering heat map of all DEGs from Figure 3.9, n=3. B) qPCR validation of 4 DEGs, 2 upregulated and 2 downregulated, identified from “A”, n=4-6. C) Top five biological processes determined by Gene Ontology analysis of all DEGs from “A” using HumanCyc 2016 (top), KEGG 2019 (middle) and Wikipathways 2019 (bottom), n=3.

Regulator	P-value	Combined score
ESR1	0.0005164	57.23
ATF3	0.001137	16.02
P300	0.004133	12.3
SMARCA4	0.004633	14.92
MECOM	0.00918	9.95
CDX2	0.01108	9.32
ATF3	0.01108	9.32
SOX2	0.01317	8.15
FOXO3	0.01507	12.49
SPI1	0.01731	7
PPAR	0.02039	9.06
EWS	0.02518	11.06
JUN	0.0271	6.84
CRX	0.0271	6.84
OCT4	0.0271	6.84
SOX6	0.0271	6.84
RXR	0.0271	6.84
SA1	0.0271	6.84
TCF12/HEB	0.0271	6.84
CTCF	0.0271	6.84

Figure 3.11 Potential Upstream Regulators of Nuclear-Parkin Responsive Genes in Response to Hypoxia. EnrichR output showing the top 20 most significantly enriched transcriptional regulators associated with the differentially expressed genes (DEGs) from Figure 3.10, nuclear receptors and cofactors emphasized in bold, red text.

CHAPTER 4: NUCLEAR PARKIN INCREASES TRANSCRIPTION OF ERR α

TARGETS

4.1 Introduction

Although Parkin has the potential to ubiquitinate many proteins, the number of validated Parkin substrates is relatively low. Because Parkin is mainly studied for its role at the mitochondria, the majority of known substrates are mitochondrial proteins. Recently, there has been a shift in focus towards identifying and validating additional substrates of Parkin—both at the mitochondria, and in other cellular compartments. I previously showed that Parkin localizes to the nucleus under baseline conditions, and its nuclear localization is dynamic and dependent upon cellular stress conditions (Chapter 2). I also showed that nuclear Parkin has wide-ranging effects on multiple cellular processes (Chapter 3). Therefore, the next logical steps include identifying specific ubiquitination targets of Parkin in the nucleus that promote these downstream effects.

In this chapter, I perform an unbiased screen for novel Parkin substrates which identifies three different nuclear proteins, including estrogen-related receptor alpha (ERR α). ERR α is an orphan nuclear receptor first identified for its structural homology to estrogen receptor (Giguere et al., 1988). ERR α , like Parkin, is robustly expressed in tissues with high metabolic demands (Huss et al., 2004; Wende et al., 2005). Although ERR α has no known endogenous ligands, studies have shown that it is activated by cholesterol (Wei et al., 2016) and the HER2 pathway (Ariazi et al., 2007). It is now well-documented that ERR α works together with a variety of coactivators, such as PGC1 α (Schreiber et al., 2004; Schreiber et al., 2003) and SIRT1 (Wilson et al., 2010) to

regulate transcription of genes related to several metabolic pathways (Audet-Walsh and Giguere, 2015). Supporting its role in metabolism, ERR α -deficient mice have reduced body weight and peripheral fat deposits compared to WT mice, and are resistant to high-fat diet-induced obesity (Luo et al., 2003). ERR α is widely studied in the context of cancer, especially breast cancer (Ariazi et al., 2002; Ranhotra, 2018). However, recent studies have shown ERR α activity is critical for functional adaptation to pressure overload in the heart (Huss et al., 2007).

Mechanistically, ERR α binds to an estrogen-related response element (ERRE) in the promoter regions of its targets to regulate their transcription (Johnston et al., 1997). Recent studies have demonstrated that, in addition to its robust role in metabolic regulation, ERR α regulates transcription of genes involved autophagy (Kim et al., 2018), innate host defense (Sonoda et al., 2007), and angiogenesis (Likhite et al., 2019). Here, I begin to investigate whether ERR α is a substrate of Parkin, and what is Parkin's effect on ERR α 's downstream stability and activity.

4.2 Results

4.2.1 Identification of ERR α as a Potential Parkin Substrate

In order to identify novel targets of Parkin, I conducted an unbiased screen by pulling down ubiquitinated proteins from heart lysates from Parkin^{-/-} and Parkin TG mice using tandem ubiquitination binding entities (TUBES)(Rubel et al., 2013) conjugated to agarose beads (Figure 4.1 A). To confirm the pulldown efficiency, I ran eluates from the pulldown along with the whole heart lysate on a western blot and immunoblotted for ubiquitin (Figure 4.1 B). As expected, Parkin TG heart lysates have increased levels of

ubiquitinated proteins compared to Parkin^{-/-} heart lysates. Additionally, the TUBE-pulldown is successfully able to enrich for ubiquitinated proteins. I therefore sent the eluates to Applied Biomix to label the samples and run them together on a 2D gel, which allowed for visualization of proteins isolated from the Parkin TG samples, but not the Parkin^{-/-} sample (Figure 4.2 A). These spots represented potential ubiquitination targets of Parkin. The spots were then picked and identified via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 4.2 B). Three of the top proteins identified were nuclear proteins: Mediator of RNA polymerase II, transcription subunit 23 (MED23), Transcription Factor EC (TFEC), and ERR α (Figure 4.2 C). I decided to pursue ERR α further as it promotes transcription of genes that increase mitochondrial biogenesis, changes in metabolism, and mitophagy (Kim et al., 2018; Schreiber et al., 2004; Singh et al., 2018).

4.2.2 Parkin Interacts with ERR α in the Nucleus

To establish whether Parkin directly interacts with ERR α , we performed a co-immunoprecipitation in cells expressing either mCherry or NLS-Parkin and either flag or flag-ERR α . NLS-Parkin co-immunoprecipitated with flag-ERR α but not flag alone, indicating the two proteins interact (Figure 4.3 A). Further, because we used NLS-Parkin, we can infer that the interaction occurs specifically in the nucleus. When we analyzed the immunoprecipitates for presence of ubiquitin, only the lane expressing both ERR α and NLS-Parkin together blotted positive for ubiquitination (Figure 4.3 B). These data collectively indicate ERR α may be a substrate of Parkin in the nucleus.

4.2.3 Parkin does not Affect ERR α Stability

Ubiquitination is a versatile process that can affect a protein's stability, subcellular localization or activity (Komander and Rape, 2012). Therefore, to determine the effect of Parkin-mediated ubiquitination of ERR α , I first tested stability. Expression of both WT Parkin and NLS-Parkin caused a modest, but significant, increase in protein levels of ERR α in cells (Figure 4.4 A and B). However, ERR α levels were not altered in hearts from Parkin KO, WT, and Parkin TG mice (Figure 4.4 C and D). To examine further whether Parkin could be affecting ERR α stability, we treated cells expressing either β -gal, WT Parkin, or NLS-Parkin with cyclohexamide to inhibit protein synthesis and assessed ERR α protein levels. We measured similar rates of ERR α degradation regardless of Parkin or NLS-Parkin expression, suggesting that Parkin does not affect ERR α stability (Figure 4.4 E and F).

4.2.4 Nuclear Parkin Increases Transcript Levels of ERR α Targets

Next, I assessed the effect of nuclear Parkin on ERR α 's activity. Functionally, ERR α binds to ERREs in the promoter regions of target genes to regulate their expression. To determine whether Parkin affects the transcriptional activity of ERR α , I assessed the mRNA levels of several known ERR α target genes via qPCR, including ACADM, ACADVL, HK1, and PPAR α . Expressing NLS-Parkin resulted in increased mRNA levels of the targets compared to mCherry at baseline conditions (Figure 4.5 A). Of note, WT Parkin expression also slightly, but not significantly increased the mRNA levels of the targets, further confirming that this effect is nuclear-Parkin specific. During hypoxia, however, transcript levels for many of the ERR α targets decreased, but not in a

Parkin-dependent manner (Figure 4.5 B). Taken together, these data indicate that nuclear Parkin increases $ERR\alpha$ transcriptional activity at baseline.

4.3 Discussion

In this chapter, I establish that Parkin interacts with transcription factor $ERR\alpha$. I also show an increase in ubiquitination when $ERR\alpha$ is immunoprecipitated in the presence of NLS-Parkin. I hypothesize that NLS-Parkin directly ubiquitinates $ERR\alpha$; however, it is also possible that Parkin and $ERR\alpha$ are in a complex together and the ubiquitinated proteins are the additional proteins within the complex. Future studies in the lab will address whether Parkin directly ubiquitinates $ERR\alpha$.

Additionally, the data presented here indicate that NLS-Parkin increases transcription of $ERR\alpha$ targets. In this model, Parkin-mediated ubiquitination increases $ERR\alpha$ activity to promote transcription of its gene targets. Ubiquitination can elicit several downstream consequences which are executed by effector proteins with ubiquitin binding domains or motifs (Dikic et al., 2009). $ERR\alpha$'s transcriptional activity is modulated by cofactors such as $PGC1\alpha$. It is therefore likely that ubiquitination of $ERR\alpha$ recruits one or more cofactor(s) in order to positively regulate its transcriptional activity.

$ERR\alpha$ has been previously reported to be a substrate of Parkin, however the study demonstrated that Parkin-mediated ubiquitination promotes degradation of $ERR\alpha$ (Ren et al., 2011). Here, I report a slight increase in $ERR\alpha$ levels in response to NLS-Parkin expression, without altered stability. As, $ERR\alpha$ has an ERRE in its own promoter, increasing its activity can lead to increased protein levels. This is one explanation for the conflicting evidence of augmented protein levels in cells expressing WT and NLS-

Parkin despite similar degradation rates. Although I measure increased transcript levels of $ERR\alpha$ targets in response to NLS-Parkin expression, I have not shown that NLS-Parkin directly increases $ERR\alpha$ transcriptional activity. Additional studies are aimed at addressing whether transcriptional changes in response to NLS-Parkin are directly mediated by increased $ERR\alpha$ activity.

Parkin's role in the nucleus, increasing transcription of $ERR\alpha$ targets, may complement its role at the mitochondria in facilitating mitochondrial clearance. Cells require functional mitochondria to generate ATP, so excessive clearance without corresponding mitochondrial biogenesis may be harmful. Increasing transcription of PGC-1 α may therefore help compensate for the loss of mitochondria, when mitophagy is activated. Additionally, there is recent evidence for $ERR\alpha$ targets involvement in mitophagy itself. For example, hexokinase activity promotes Parkin translocation to depolarized mitochondria (McCoy et al., 2014), while phosphorylation of MCAD may be important for Parkin-mediated mitophagy as it can rescue PINK1 deficiencies in behavior and metabolism (Course et al., 2018). $ERR\alpha$ has many additional transcriptional targets not assessed in this study, and future studies should determine whether nuclear Parkin also affects transcription of these targets.

Although Parkin is widely-studied for its role in mitophagy, $ERR\alpha$ is not the first transcriptional regulator shown to be a target of Parkin. Parkin ubiquitinates Parkin-interacting substrate (PARIS), which conversely represses PGC-1 α -mediated transcription, and decreases mitochondrial biogenesis (Shin et al., 2011). It is important to note that although Parkin is best known for ubiquitinating proteins for degradation, ubiquitination is an extremely versatile process, and can promote a change in a

protein's conformation, subcellular localization, as well as activity (Komander and Rape, 2012). As there is clear evidence for additional targets of Parkin in the nucleus, future research should focus on identifying these nuclear substrates of Parkin, understanding how Parkin-mediated ubiquitination affects the proteins and their transcription pathways, and determining whether they are able to be targeted therapeutically.

4.4 Materials and Methods

Animals

All animal experiments were performed according to guidelines specified by the University of California, San Diego Institutional Animal Care and Use Committee. Parkin KO mice were obtained from Jackson Laboratories (B6. 129S4-Park2tm1Shn/J, stock number 006582) and their cardiac phenotype has been characterized previously (Kubli et al., 2013b). The Parkin TG mice overexpress human Parkin under the cardiomyocyte-specific α -myosin heavy chain (α -MHC) promoter and were generated at the UCSD Mouse Transgenic Core as described previously (Woodall et al., 2019).

Cell Culture

HeLa cells were plated and maintained in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher) containing 10% fetal bovine serum (FBS, ThermoFisher) and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), and cultured in 5% CO₂ atmosphere at 37°C.

Plasmids and Adenoviral Constructs

Flag-ERR α was acquired from Addgene (10975). We generated the mCherry-Parkin virus from the plasmid (Addgene #23956). NLS-Parkin was generated by fusing mCherry linked to three consecutive SV40 nuclear localization signals (pmCherry-NLS, Addgene 39319) to the N-terminal end of Parkin. Cells were transiently transfected with cDNA using Fugene 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Cells were infected with adenoviruses in DMEM + 2% heat-inactivated serum and rescued with their respective culture media. Treatments and experiments were performed 24 hrs post-infection or -transfection.

Immunoprecipitation and Immunoblotting

HeLa cells were harvested and lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Complete protease inhibitor (Roche Applied Science). For immunoprecipitation, lysates were pre-cleared with 40 μ L protein A agarose beads (sc 2001) for 30 minutes at 4°C and subjected to immunoprecipitation with anti-Flag (Sigma, F7425). 4 μ g of antibody was added to 1.5 μ g of cell lysate and incubated at 4°C for 18 hours. After incubation for 1.5 hrs with protein A agarose beads, immunoprecipitates were washed with ice-cold PBS and eluted with SDS loading buffer by boiling for 5 minutes at 95°C. For immunoblotting, samples were run on NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were immunoblotted with the following antibodies: Parkin (Cell Signaling Technology), ERR α (Abcam), flag (Sigma, F1804), Histone H3 (Cell Signaling Technology), GAPDH

(GeneTex), Ubiquitin (Santa Cruz Biotechnology) and imaged using a ChemiDoc XRS+ System (Bio-Rad).

Quantitative PCR

RNA extraction, genomic DNA extraction, cDNA synthesis, and quantitative PCR were performed as described previously (Orogo et al., 2015). Briefly, genomic DNA and total RNA were isolated from cells using the GenElute Mammalian Genomic DNA Miniprep Kit (MilliporeSigma) and the RNeasy Mini Kit (Qiagen), respectively, following the manufacturers protocols. cDNA was synthesized according to the manufacturer's protocol using the QuantiTect Reverse Transcription kit (Qiagen). TaqMan primers for *Ppargc1a*, *ESRRA*, *PPARA*, *ACADM*, *FABP3*, *HK1*, *ACADVL*, *HMGB2*, and *Rn18s* and TaqMan Universal Master Mix II was purchased from Applied Biosystems/Life Technologies. Primer sequences are available in Table S1. qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Relative mRNA was normalized to *Rn18s*, and fold change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

TUBE-Pulldown and Target Identification

These experiments were carried out as previously described (Rubel et al., 2013). Briefly, whole heart lysates were made fresh from Parkin-null and Parkin TG mice, pre-cleared with unconjugated agarose beads, and combined with agarose beads bound to tandem ubiquitin binding entities (TUBES, LifeSensors), and pulled down via centrifugation. An additional Parkin TG lysate pre-cleared, and then pulled with unconjugated agarose beads was also added as a control condition. Proteins were

subsequently eluted from the beads and sent to (Applied Biomics) to be labeled with dye, run on a 2-D gel, and spots picked for identification via liquid chromatography, tandem mass spectrometry.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Graphed data were represented as mean $\pm$ SEM. Unpaired Student's t test was used for two-group comparisons. One- or two-way ANOVA with post hoc comparisons by Tukey's method was used for the comparisons between multiple groups.

Parts of Chapter 4 were submitted for publishing. Shires, S. E., Quiles, J. M., Najor, R. H., Leon, L. J., Cortez, M. Q., Lampert, M. A., Mark, A., Gustafsson, Å. B. Nuclear Parkin Promotes the ERR α -mediated Transcriptional Program and Drives Widespread Changes in Gene Expression Following Hypoxia. The dissertation author was the primary investigator and author of this paper.

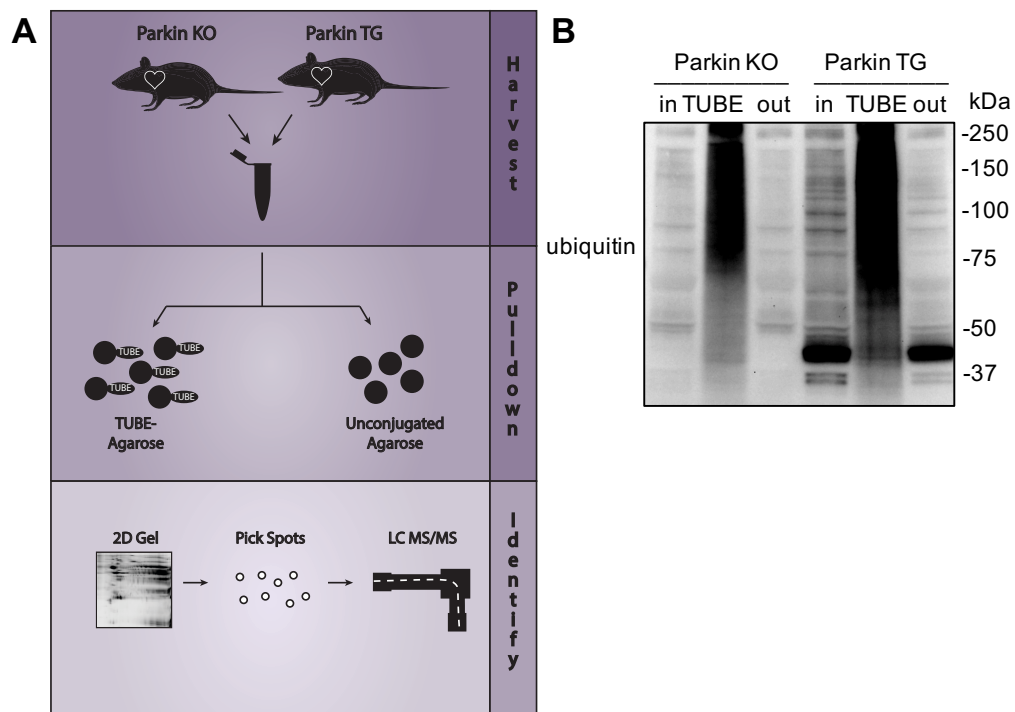


Figure 4.1 Unbiased Screen for Novel Parkin Substrates. A) Diagram of experimental setup: Lysates were generated from the hearts of Parkin KO and Parkin TG mice, pre-cleared with unconjugated agarose beads, and ubiquitinated proteins were pulled down by incubation with TUBE-agarose beads and subsequent centrifugation and washes. Immunoprecipitates were eluted from beads and run on a 2-D gel to separate the protein. Spots representing proteins pulled down in the Parkin TG sample that were not in the Parkin KO sample were isolated and proteins identified via LC-MS/MS. B) Representative immunoblot of ubiquitin from whole heart lysate input labeled “pre”, pull-downs labeled “TUBE”, and lysates post pull-down labeled “out”.

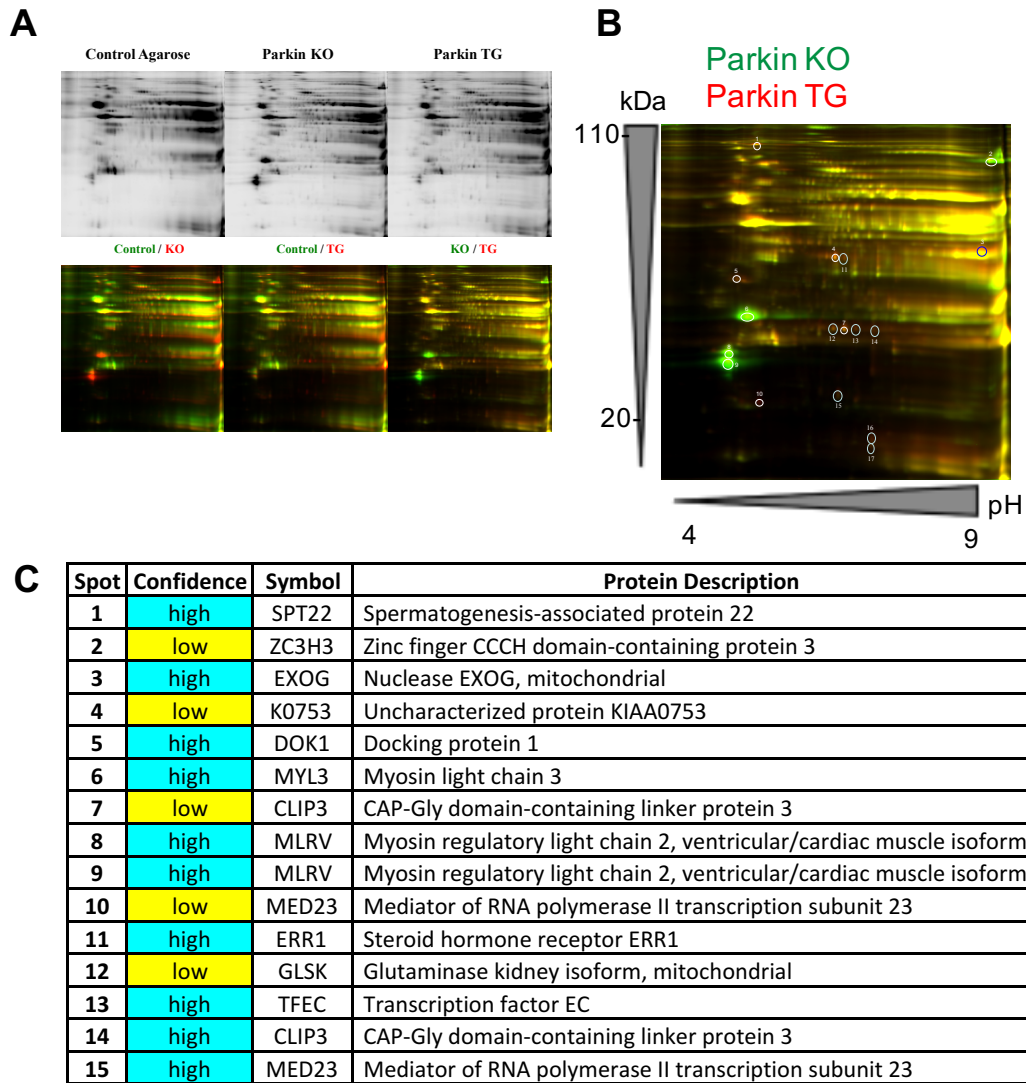


Figure 4.2 Identification of ERR α as a Potential Parkin Substrate. A) 2-D gel images comparing pull-downs from control, Parkin^{-/-} lysate, and Parkin-TG lysate. B) 2-D gel image of separated proteins from immunoprecipitates from Parkin^{-/-} vs Parkin TG heart lysates. Circles highlight spots picked for identification. C) Chart with proteins identified via LC-MS/MS.

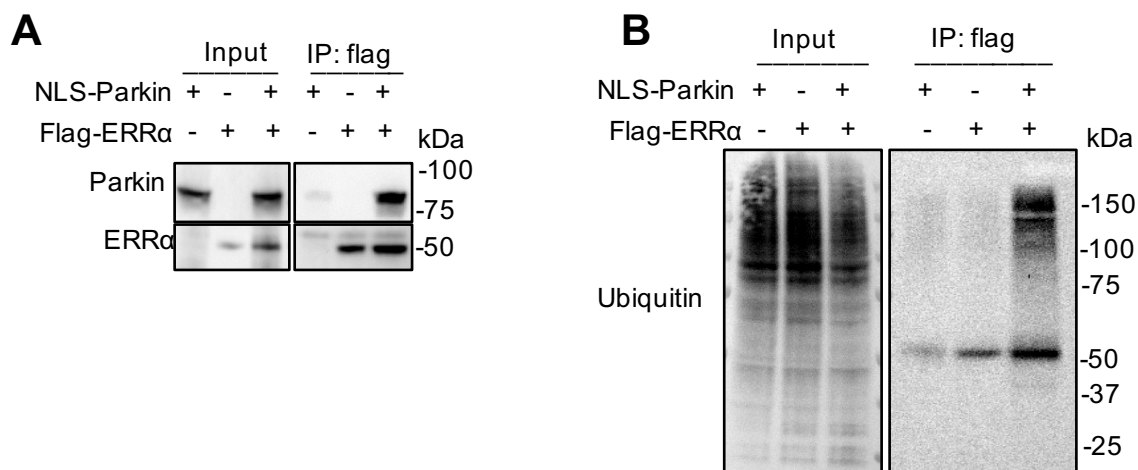


Figure 4.3 Parkin interacts with ERR α in the Nucleus. A) Representative immunoblot of protein levels of Parkin and ERR α before (left) and after co-immunoprecipitation of NLS-Parkin with ERR α using anti-flag (right). B) Representative immunoblot of ubiquitin levels before (left) and after co-immunoprecipitation of NLS-Parkin with ERR α using anti-flag (right).

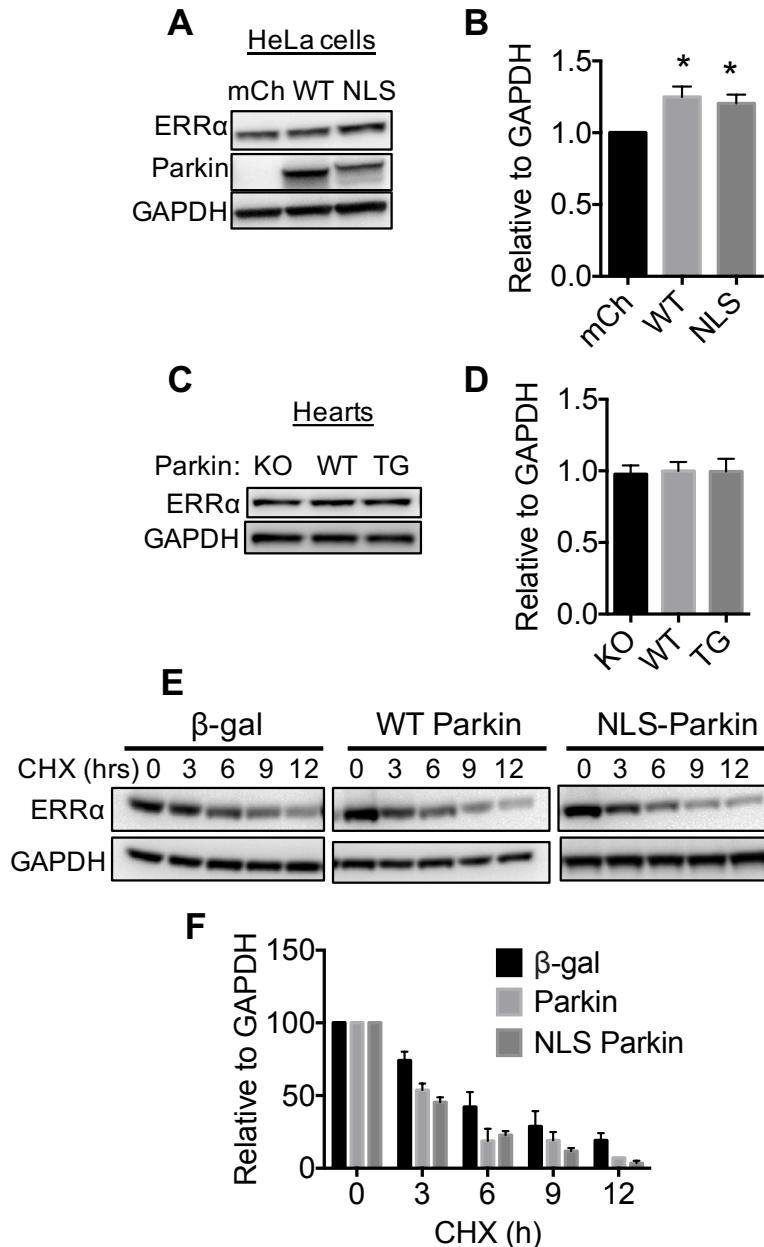


Figure 4.4 Parkin does not Affect ERRα Stability. A) Representative immunoblot of protein levels of endogenous ERRα in HeLa cells expressing mCherry, WT Parkin, or NLS-Parkin. B) Quantification of ERRα levels relative to GAPDH in HeLa cells expressing mCherry, WT Parkin, or NLS-Parkin, n=5. C) Representative immunoblot of ERRα protein levels in hearts from Parkin KO, WT, or Parkin-TG mice. D) Quantification of ERRα levels relative to GAPDH in hearts from Parkin KO, WT, or Parkin-TG mice, n=3. E) Representative immunoblot of protein levels of ERRα in HeLa cells expressing β-gal, WT Parkin, or NLS-Parkin and subjected to cyclohexamide (CHX) for indicated time points. F) Quantification of ERRα levels relative to GAPDH in HeLa cells expressing β-gal, WT Parkin, or NLS-Parkin and subjected to cyclohexamide for indicated time points, n=3, *p,0.05 compared to mCh.

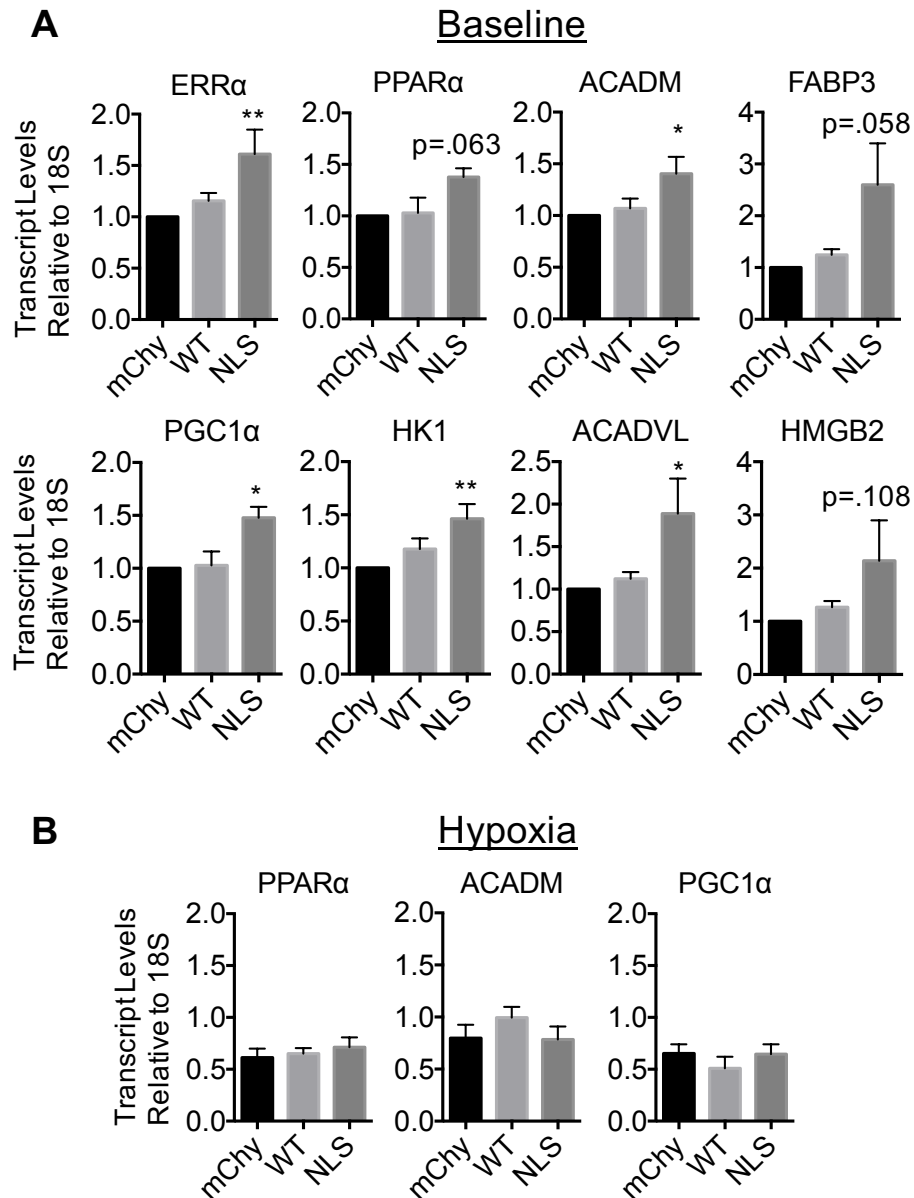


Figure 4.5. Nuclear Parkin activates ERRα-regulated gene transcription. A) Transcript levels of ERRα targets in HeLa cells expressing either mCherry, WT Parkin, or NLS-Parkin, n=3-6, *p<0.05 compared to mCherry. **B)** Transcript levels of ERRα targets in HeLa cells expressing either mCherry, WT Parkin, or NLS-Parkin and subjected to hypoxia for 12 hours, n=3-6, *p<0.05 compared to mCherry. All mRNA levels were normalized to mCherry normoxia.

CHAPTER 5: OVEREXPRESSION OF PARKIN DOES NOT RESCUE PRESSURE OVERLOAD-INDUCED CARDIAC HYPERTROPHY

5.1 Introduction

Heart failure can develop as a result of an acute cardiac injury, such as a myocardial infarction (MI), or in response to chronic pressure-overload, such as high blood pressure. Both acute and chronic cardiac stressors have been associated with mitochondrial damage (Faerber et al., 2011; Lesnefsky et al., 2001). Recent research has focused on determining the roles of autophagy and mitophagy in heart disease development and cardioprotection. A recent study assessing autophagy versus mitophagy in a model of pressure-overload-induced heart failure has shown that general autophagy is rapidly upregulated within hours, but then chronically downregulated after 5 days, while mitophagy is transiently activated within 3-7 days, but then downregulated thereafter (Shirakabe et al., 2016).

General autophagy can be both protective and harmful when activated during heart disease (Nakai et al., 2007; Zhu et al., 2007). Several studies have shown a protective effect of Parkin-mediated mitophagy in the heart. Although Parkin-null mice have normal cardiac function when young (Kubli et al., 2013b), they accumulate abnormal mitochondria with age (Hoshino et al., 2013; Kubli et al., 2013a). Additionally, Parkin-deficient mice are more susceptible to both acute cardiac injury and chronic cardiac stress. Parkin-null mice develop severe cardiac dysfunction and increased mortality compared to WT mice in response to MI (Kubli et al., 2013b). Parkin-deficiency also increases cardiac hypertrophy and contractile dysfunction in response to chronic

pressure-overload (Han et al., 2017). Furthermore, Parkin is critical for cardiac metabolic maturation (Gong et al., 2015) and ischemic pre-conditioning (Huang et al., 2011). These data highlight the importance of Parkin in responding to metabolic maturation, stress, and aging.

Overexpression of Parkin has been shown to reduce age-associated cardiac dysfunction and preserve mitochondrial function in mice (Hoshino et al., 2013). However, it is unclear whether enhancing Parkin-mediated mitophagy would be protective or detrimental during cardiac pressure-overload. In this chapter, I examine the effect of overexpressing Parkin in a cardiac pressure-overload model, trans-aortic constriction (TAC). I found that overexpressing Parkin in the heart does not alter mitochondrial content for up to two weeks in response to TAC. Additionally, Parkin TG mice develop perivascular fibrosis at a slower rate than WT mice in response to TAC. However, increased Parkin expression did not have an effect on development of cardiac hypertrophy or dysfunction in response to TAC.

5.2 Results:

5.2.1 Mitochondrial Content and Function are Unchanged in the Hearts of WT and Parkin TG mice in Response to TAC

In order to determine the role of Parkin in adapting to chronic cardiac stress, I subjected WT and Parkin TG mice to TAC. TAC decreases the radius of the aorta, thus increasing the resistance and workload for the heart, mimicking the pathogenesis of heart failure induced by high blood pressure (deAlmeida et al., 2010; Rockman et al., 1991). Because Parkin's role in cardioprotection is mainly attributed to its role in

clearing damaged mitochondria, I first measured mitochondria levels in Parkin TG mice compared to WT mice at baseline and in response to TAC. Western blotting for levels of OXPHOS proteins revealed two findings: 1. mitochondrial content remains relatively stable in response to TAC in WT mice, and 2. increasing cardiac Parkin levels does not affect mitochondria levels during TAC (Figure 5.1 A and B). As mitochondrial protein levels can vary without changing the number of mitochondria, I confirmed my findings through qPCR of mtDNA compared to genomic DNA (Figure 5.1 C). Taken together, my data suggest that mitochondrial content is relatively unchanged in the hearts of WT and Parkin TG mice in response to up to two weeks of TAC.

Although I did not detect a decrease in mitochondrial content, mitophagy may still be increased if mitochondrial biogenesis is similarly augmented. PGC1 α and PGC1 β are transcriptional coactivators that play a central role in regulating mitochondrial biogenesis (Scarpulla, 2011). To determine whether mitochondrial biogenesis was altered in the hearts of Parkin TG mice in response to TAC, I assessed transcript levels of PGC1 α and PGC1 β at various time points up to 14 days post-TAC (Figure 5.2). I did not measure any differences in transcript levels in either PGC1 α or PGC1 β in response to TAC. Because Parkin TG mice have similar mitochondrial levels at baseline and during TAC, and I did not detect an increase in mitochondrial biogenesis, these data suggest that Parkin TG mice do not display greater mitochondrial turnover compared to WT mice.

During heart failure, the cardiac mitochondria undergo a maladaptive process called metabolism switching, whereby they begin utilizing glucose instead of fatty acids to generate ATP (Stanley et al., 2005; Taegtmeyer et al., 2010). Therefore, to assess

whether mitochondrial function and substrate utilization are altered between WT and Parkin TG mice, we tested oxygen consumption rate of mitochondria isolated from hearts of WT and Parkin TG mice at baseline and two weeks post-TAC. Complex I-dependent oxygen consumption rates in cardiac mitochondria were similar between WT and Parkin TG mice both at baseline and in response to TAC when utilizing pyruvate/malate and palmitoyl carnitine (Figure 5.3 A and B). However, Parkin TG cardiac mitochondria had an increased rate of Complex II-dependent oxygen consumption at two weeks post-TAC utilizing succinate when Complex I was inhibited by rotenone (Figure 5.3 C). I also examined transcript levels of various regulators of metabolism (Figure 5.4). Interestingly, I measured an increase in $ERR\alpha$ levels 2-weeks post-TAC in Parkin TG hearts compared with WT hearts. However, although transcription of $ERR\alpha$ was increased, I did not detect increased mRNA levels of its transcriptional targets, $ACADM$, $CD36$, and $PPAR\alpha$ (Figure 5.4). Taken together, these data indicate that Parkin TG mice have similar mitochondrial number compared with WT mice in healthy and pressure-overloaded hearts, but that metabolic regulation and Complex II activity is increased in Parkin TG cardiac mitochondria in response to TAC.

5.2.2 Parkin TG mice Develop Perivascular Fibrosis at a Slower rate than WT mice in Response to TAC

During chronic pressure-overload, hearts experience a loss of myocytes that cause fibroblast infiltration and permanent fibrotic damage. To assess cardiac fibrosis, I stained sections from hearts harvested under baseline conditions and at 2 and 8 weeks post-TAC with Masson's trichrome (Figure 5.5). Interestingly, although I did not see any

major increases in total fibrosis or interstitial fibrosis, I noticed an increase in fibrosis around the vessels (perivascular fibrosis) at 2 weeks post-TAC in WT hearts that was not mirrored in Parkin TG hearts (Figure 5.5 B and D). However, by 8 weeks post-TAC, perivascular fibrosis looked similar between the WT and Parkin TG hearts (Figure 5.5 C and D). Taken together, these data indicate that Parkin TG mice develop perivascular fibrosis at a slower rate than WT mice in response to TAC.

5.2.3 Parkin Overexpression has no Effect on Cardiac Function or Hypertrophy in Response to TAC

Finally, I assessed whether enhanced levels of Parkin in the heart would protect against TAC-induced heart failure. First, I assessed cardiac function via echocardiogram at baseline and every two weeks until eight weeks post-TAC. To confirm surgical success, I measured blood flow velocity across the aortic arch, and if it did not increase at least 2.5-fold, the mouse was removed from the study (Figure 5.6 A). Notably, I did not detect differences in cardiac function, measured by fractional shortening (FS) and ejection fraction (EF) between WT and Parkin TG hearts (Figure 5.6 B and C). Furthermore, I did not observe any differences in systolic or diastolic dimensions or LV mass between WT and Parkin TG mice in response to TAC (Table 5.1), suggesting that overexpression of Parkin neither sensitizes nor rescues cardiac dysfunction in response to pressure-overload. I confirmed that Parkin TG mice do not develop hypertrophy more rapidly via qPCR for hypertrophy markers, atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) (Figure 5.7 A and B). As expected, although hearts from both WT and Parkin TG mice develop increases in ANF and BNP

in response to TAC, there was no effect of Parkin overexpression on expression of hypertrophy markers. Taken together, these data reveal that both WT and Parkin TG mice develop cardiac hypertrophy and dysfunction in response to TAC and that cardiac-specific overexpression of Parkin neither sensitizes nor rescues these developments.

5.3 Discussion

In this chapter, I report several new findings on the role of Parkin during chronic cardiac pressure-overload. First, I determined that increased expression of Parkin during chronic pressure overload has no effect on mitochondrial quantity. Mitochondrial protein levels and mtDNA content remained relatively constant over the course of the first two weeks of TAC in hearts from both WT and Parkin TG mice. I also did not detect altered mitochondrial biogenesis in hearts from WT and Parkin TG mice in response to TAC. However, Parkin overexpression does alter Complex II activity and metabolic gene transcription in response to TAC.

Another interesting finding is that Parkin TG hearts developed perivascular fibrosis at a slower rate than WT mice. Development of fibrosis in response to cardiac pressure overload is a complex process, the mechanisms of which are poorly understood (Kong et al., 2014). The endothelium is thought to play a central role in the development of cardiac fibrosis; however, Parkin TG mice overexpress Parkin solely in myocytes. Future studies should examine whether cardiac myocytes create signals that promote fibrosis, and whether Parkin plays a role in signal generation or propagation.

Although I measured an increase in $ERR\alpha$ transcript levels in Parkin TG hearts in response to TAC, I did not observe an increase in mRNA levels of its transcriptional

targets. This suggests that $ERR\alpha$ may have been increased, but was not active—at least at the two-week post-TAC time point. Future studies should be extended to a later time point to determine whether $ERR\alpha$ -target transcript levels increase. Additionally, transcriptional profiling of Parkin-overexpressing hearts in heart failure may help identify additional transcriptional alterations metabolic genes, as well as identify potential mechanisms for both the metabolic and fibrotic differences in Parkin TG hearts in response to TAC.

5.4 Materials and Methods

Animals

All animal experiments were performed according to guidelines specified by the University of California, San Diego Institutional Animal Care and Use Committee. Parkin TG mice overexpress human Parkin under the cardiomyocyte-specific α -myosin heavy chain (α -MHC) promoter and were generated at the UCSD Mouse Transgenic Core as described previously (Woodall et al., 2019).

Transverse Aortic Constriction

Mice were anesthetized with isoflourane, intubated and ventilated. A partial upper sternotomy was performed to access the aortic arch. To constrict the aorta, a 7-0 silk suture was threaded underneath the aortic arch and tied around the arch and a 27-gauge blunt-tipped needle between the left carotid and brachiocephalic arteries and the needle removed. The ribs were rejoined with a 4-0 silk suture, air massaged out of the

chest, and the incision site closed using surgical glue. Successful surgery was confirmed via echocardiogram of pressure across the aortic arch.

Echocardiography

Echocardiography was performed as described previously (Woodall et al., 2019). Mice were lightly anesthetized through a nose cone and placed on a warming pad. Heart function was imaged with the Vevo770 In Vivo Micro-Imaging System with an RMV707B 15–45 MHz imaging transducer (VisualSonics Inc.) and analyzed using VisualSonics software.

Histology

Hearts were fixed in 10% formalin for 20-24 hours, transferred to 70% ethanol for 1 week, embedded in paraffin, and sectioned in 10 μ m cross-sections using a microtome (Leica Biosystems). Sections were stained with Masson's Trichrome (MilliporeSigma). Percent fibrosis was determined through ImageJ quantification of at least five images per section, covering the entire left ventricular area.

Immunoblotting

Hearts were harvested and homogenized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and Complete protease inhibitor (Roche Applied Science). Samples were run on NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were immunoblotted with the following antibodies: Parkin (Cell

Signaling Technology), MitoProfile Total OXPHOS Rodent WB antibody cocktail (MitoSciences), Actin (Sigma), and GAPDH (GeneTex), and imaged using a ChemiDoc XRS+ System (Bio-Rad).

Quantitative PCR

RNA extraction, genomic DNA extraction, cDNA synthesis, and quantitative PCR were performed as described previously (Orogo et al., 2015). For RNA isolation from the heart, slices were removed from the center of the ventricle and incubated in RNAlater solution (Ambion) for 24-48 hrs before flash-freezing. Total RNA was extracted through the Qias shredder and RNeasy Fibrous Tissue Mini Kits (Qiagen). Mitochondrial and genomic DNA were isolated using the GenElute Mammalian Genomic DNA Miniprep Kit (MilliporeSigma). cDNA was synthesized according to the manufacturer's protocol using the QuantiTect Reverse Transcription kit (Qiagen). TaqMan primers for *Ppargc1α/β*, *ESRRA*, *ACADM*, *CD36*, *PPARα*, *ANF*, *BNP*, *D-Loop* (mtDNA), *GAPDH*, and *Rn18s* and TaqMan Universal Master Mix II was purchased from Applied Biosystems/Life Technologies. qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Relative mRNA was normalized to *GAPDH* or *Rn18s*, and fold change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Mitochondrial Respiration

Hearts were homogenized using a Polytron followed by a Potter-Elvehjem Teflon tissue grinder in buffer containing 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, and 0.5% fatty acid-free BSA. The homogenate was then centrifuged at 500

x g for 10 minutes at 4°C to pellet unlysed cells and the supernatant was centrifuged at 12,000 x g for 10 minutes at 4°C to obtain a mitochondrial pellet. The pellet was washed twice in buffer, centrifuged at 12,000 g for 10 mins at 4°C, and resuspended in ~20 µL of buffer. Mitochondria were plated on a 96-well microplate at 0.5 µg or 1 µg per well. Assay media (70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA, 0.2% BSA, and 4 mM ADP) containing different substrates was used to assess glucose metabolism (10 mM pyruvate and 1 mM malate), fatty acid oxidation (40 µM palmitoylcarnitine and 1 mM malate), and complex II-dependent oxygen consumption (10 mM succinate and 2 µM rotenone). Oligomycin (2 µM) was added to inhibit ATP synthase followed by 3 successive additions of 1.5 µM FCCP to obtain maximal respiration rate. Data were analyzed using Wave for Desktop (Seahorse Bioscience – Agilent Technologies).

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Graphed data were represented as mean +/- SEM. Unpaired Student's t test was used for two-group comparisons. One- or two-way ANOVA with post hoc comparisons by Tukey's method was used for the comparisons between multiple groups.

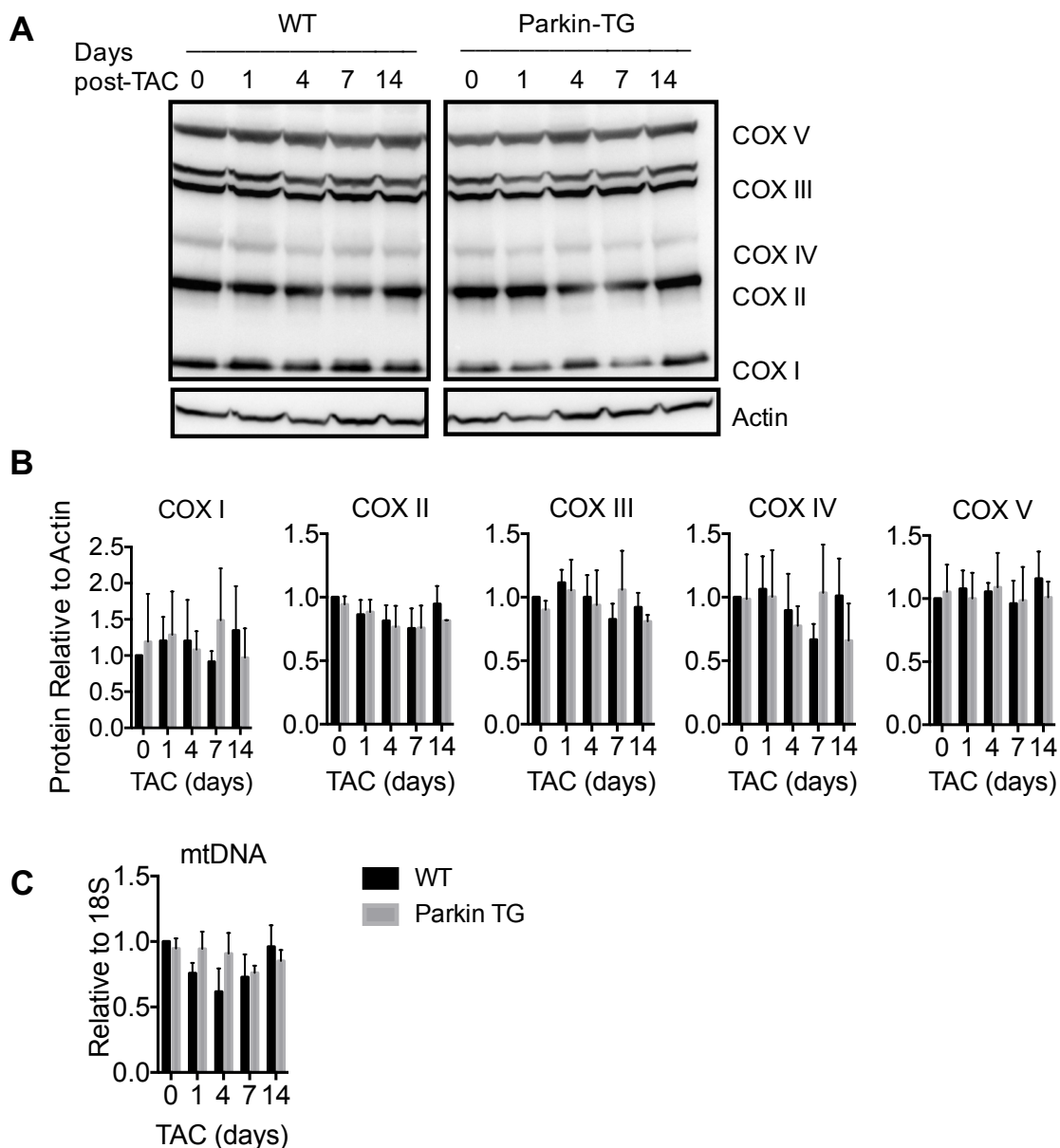


Figure 5.1 Mitochondrial Content is Similar in the Hearts of Parkin TG mice compared to WT in Response to two Weeks of TAC. A) Representative immunoblot of protein levels of OXPHOS proteins in hearts from WT and Parkin TG mice in response to up to 14 days of TAC. Immunoblot is representative of n=3. B) Quantification of OXPHOS protein levels relative to Actin in hearts from WT and Parkin TG mice in response to up to 14 days of TAC, n=3. C) Quantification of mtDNA relative to 18S in hearts from WT and Parkin TG mice in response to up to 14 days of TAC, n=4-5.

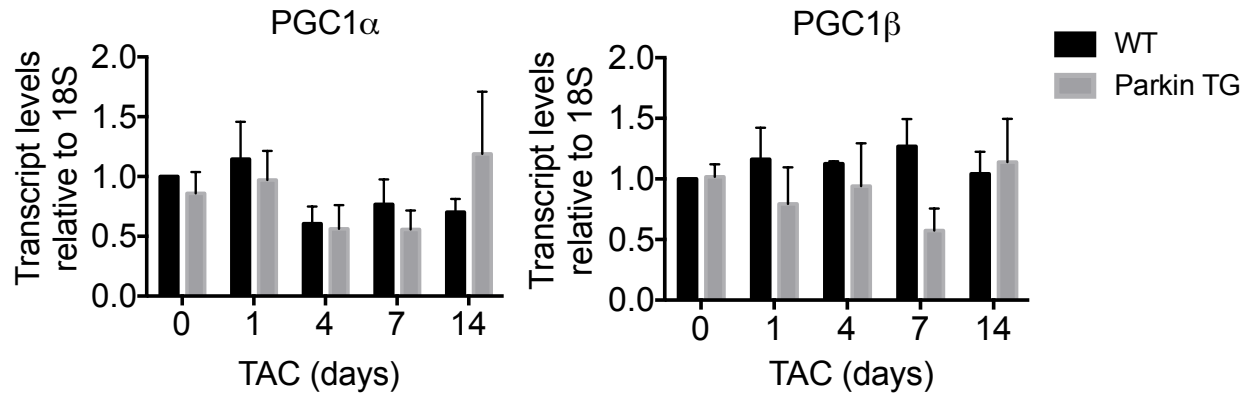


Figure 5.2 PGC1 α and PGC1 β Transcript Levels are Similar in Hearts of WT and Parkin TG mice in Response to 2 Weeks of TAC. Quantification of transcript levels of PGC1 α (left) and PGC1 β (right) relative to 18S in hearts from WT and Parkin TG mice in response to up to 14 days of TAC, n=4-5.

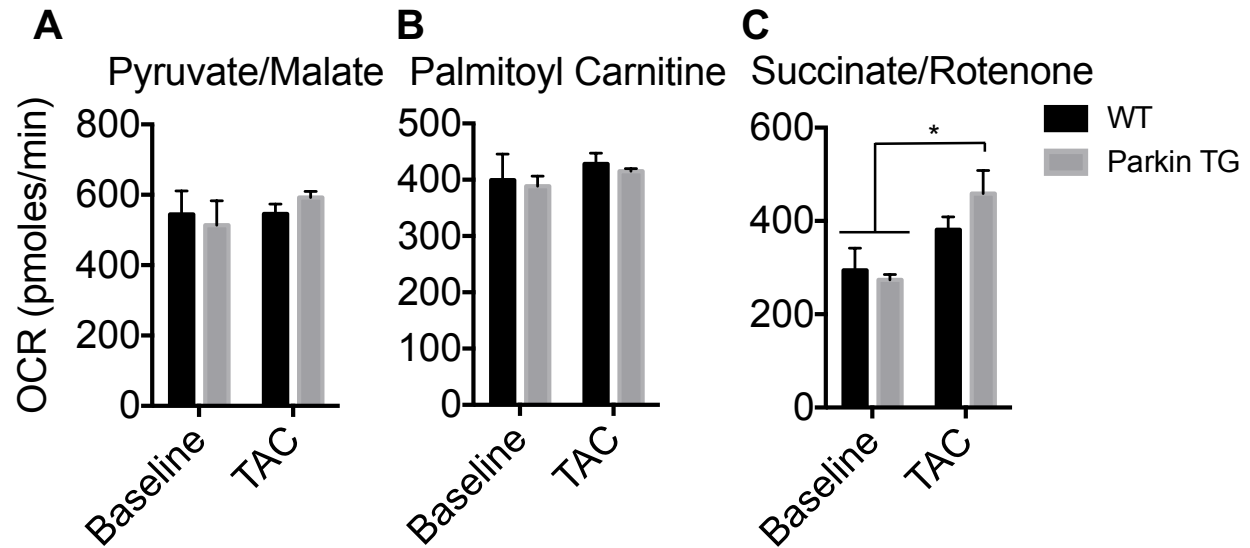


Figure 5.3 Mitochondrial Respiration is Similar in the Hearts of WT and Parkin TG mice in Response to 2 weeks of TAC. Oxygen consumption rates (OCR) of mitochondria from hearts of WT and Parkin TG mice at baseline and in response to 2 weeks of TAC with Pyruvate and Malate (A), Palmyitoyl Carnitine (B), and Succinate (C) as substrates. n=3-4, *p<0.05.

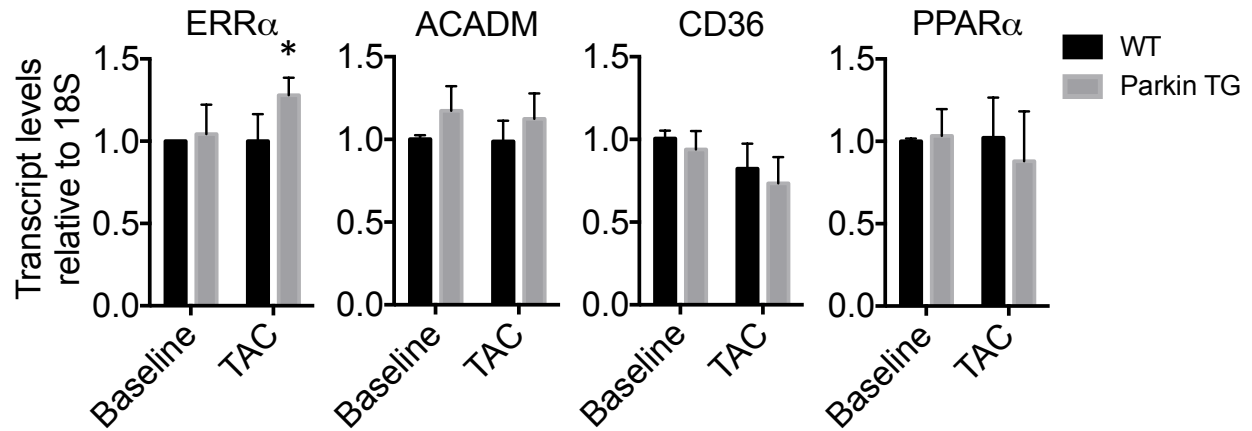
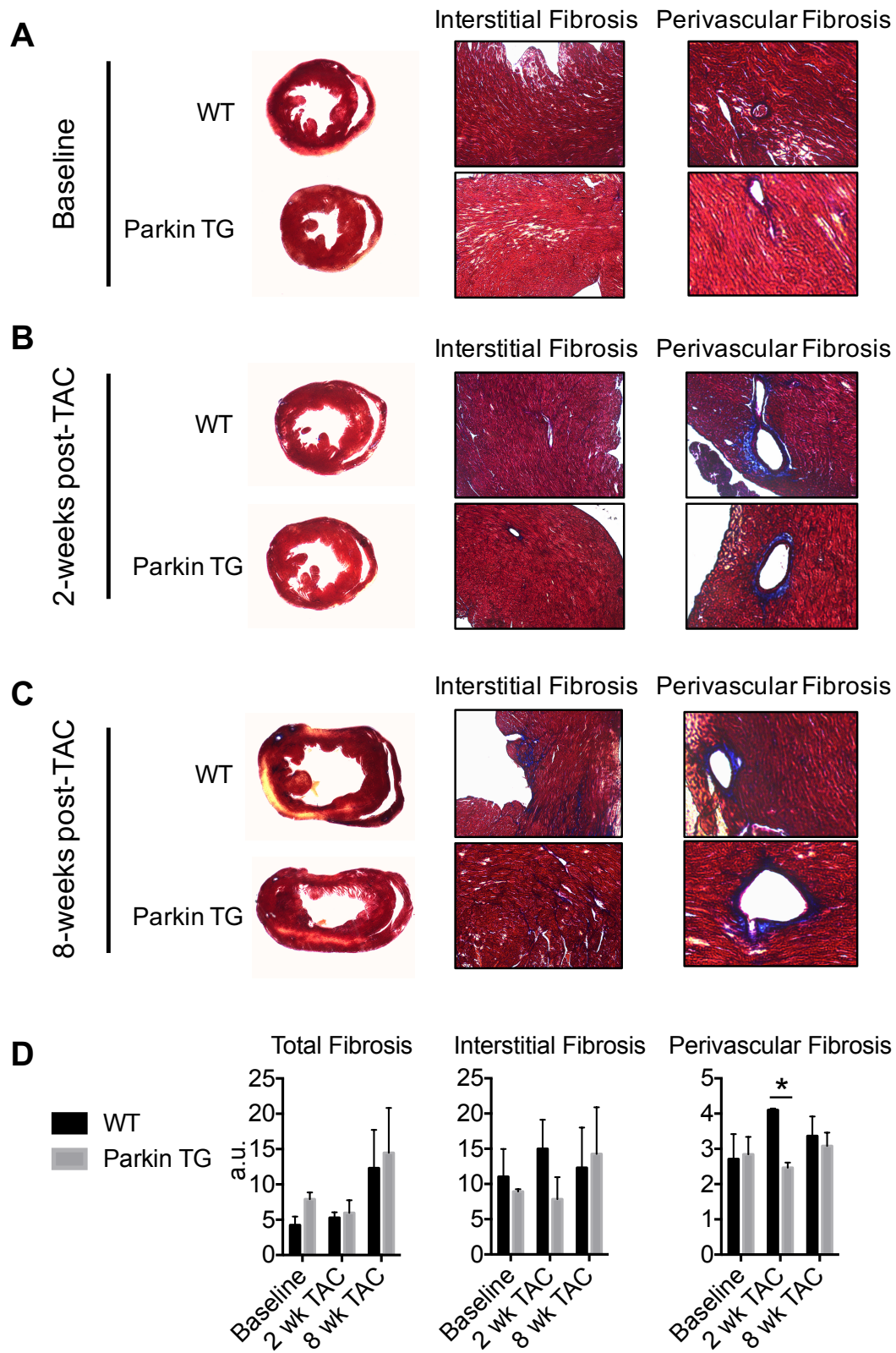


Figure 5.4 Transcript Levels of ERR α , but not its Transcriptional Targets, are Increased at two Weeks Post-TAC in Hearts of Parkin TG mice. Transcript levels of ERR α , ACADM, CD36, and PPAR α relative to 18S in hearts of WT and Parkin TG mice at baseline and in response to two weeks of TAC. n=8-9, *p<0.05

Figure 5.5 Parkin TG mice Develop less Perivascular Fibrosis than WT mice at two Weeks Post-TAC. A) Representative Masson's trichrome staining of WT and Parkin TG hearts at baseline. Whole heart cross-section (left), interstitial fibrosis (middle), and perivascular fibrosis (right). Images are representative of n=3-6 hearts. B) Representative Masson's trichrome staining of WT and Parkin TG hearts at two weeks post-TAC. Whole heart cross-section (left), interstitial fibrosis (middle), and perivascular fibrosis (right). Images are representative of n=3-6 hearts. C) Representative Masson's trichrome staining of WT and Parkin TG hearts at eight weeks post-TAC. Whole heart cross-section (left), interstitial fibrosis (middle), and perivascular fibrosis (right). Images are representative of n=3-6 hearts. D) Quantification of total (left), interstitial (middle), and perivascular (right) fibrosis in hearts from WT and Parkin TG mice at baseline, two weeks, and eight weeks post-TAC. n=3-6, *p<0.05 compared to WT.



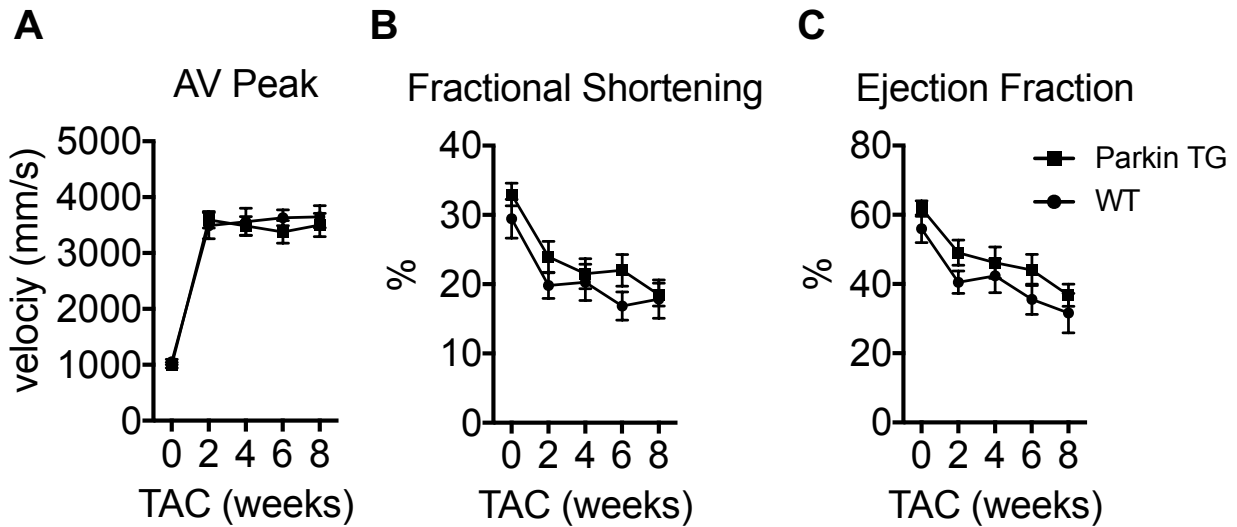


Figure 5.6 Parkin Overexpression has no Effect on Cardiac Function in Response to TAC. A) Quantification of peak aortic velocity (AV Peak) at baseline and in response to up to eight weeks of TAC in WT and Parkin TG mice, n=8-14 mice per group. B) Quantification of percent (%) fractional shortening at baseline and in response to up to eight weeks of TAC in WT and Parkin TG mice, n=8-14 mice per group C) Quantification of percent (%) ejection fraction at baseline and in response to up to eight weeks of TAC in WT and Parkin TG mice, n=8-14 mice per group.

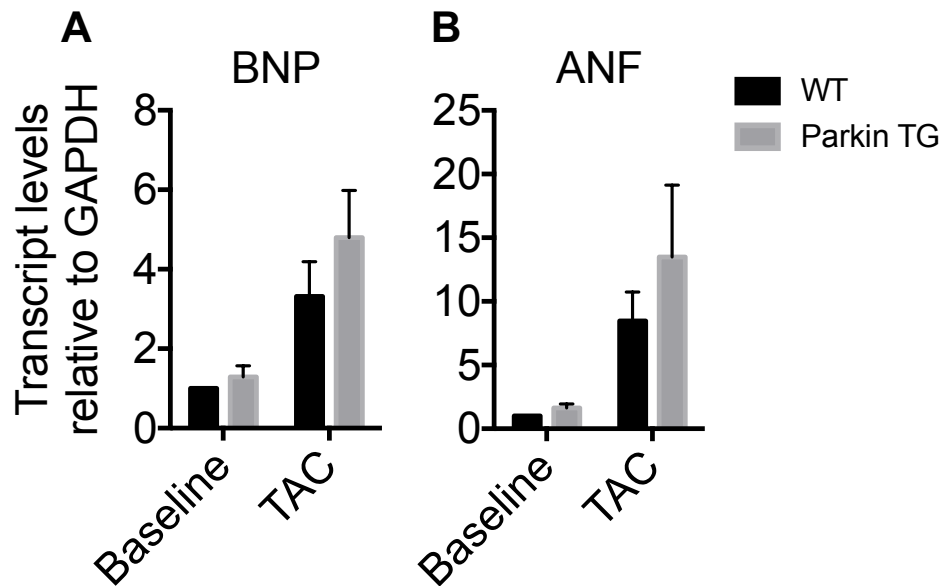


Figure 5.7 BNP and ANF Transcript Levels are Similar in Hearts of WT and Parkin TG mice in Response to two Weeks of TAC. A) Quantification of transcript levels of brain natriuretic peptide (BNP) relative to GAPDH in hearts from WT and Parkin TG mice at baseline and in response to two weeks of TAC, n=8-9 hearts per group. B) Quantification of transcript levels of atrial natriuretic factor (ANF) relative to GAPDH in hearts from WT and Parkin TG mice at baseline and in response to up to eight weeks of TAC, n=8 hearts per group.

Table 5.1 Echocardiographic analysis of WT and Parkin TG mice at baseline and in response to TAC

Table 5.1: Echocardiographic analysis of WT and Parkin TG mice at baseline and in response to TAC

	Mean $\pm$ SEM													
	Baseline				2 weeks				4 weeks				6 weeks	
	WT (n=9)	Parkin TG (n=14)	WT (n=9)	Parkin TG (n=14)	WT (n=14)	Parkin TG (n=14)	WT (n=9)	Parkin TG (n=13)	WT (n=8)	Parkin TG (n=13)	WT (n=8)	Parkin TG (n=12)	WT (n=8)	Parkin TG (n=12)
HR	458 $\pm$ 12	449 $\pm$ 6	463 $\pm$ 19	463 $\pm$ 19	455 $\pm$ 19	455 $\pm$ 19	504 $\pm$ 18	487 $\pm$ 15	480 $\pm$ 26	484 $\pm$ 16	522 $\pm$ 22	504 $\pm$ 24	522 $\pm$ 22	504 $\pm$ 24
IVSd	0.74 $\pm$ 0.05	0.80 $\pm$ 0.04	0.93 $\pm$ 0.07	0.93 $\pm$ 0.07	0.90 $\pm$ 0.06	0.90 $\pm$ 0.06	0.72 $\pm$ 0.05	0.93 $\pm$ 0.05	0.89 $\pm$ 0.04	0.84 $\pm$ 0.05	0.88 $\pm$ 0.04	0.98 $\pm$ 0.05	0.88 $\pm$ 0.04	0.98 $\pm$ 0.05
IVSs	1.04 $\pm$ 0.05	1.08 $\pm$ 0.07	1.12 $\pm$ 0.08	1.12 $\pm$ 0.08	1.23 $\pm$ 0.06	1.23 $\pm$ 0.06	1.00 $\pm$ 0.06	1.18 $\pm$ 0.06	1.13 $\pm$ 0.05	1.16 $\pm$ 0.07	1.12 $\pm$ 0.07	1.24 $\pm$ 0.04	1.16 $\pm$ 0.07	1.24 $\pm$ 0.04
LVPWd	0.73 $\pm$ 0.08	0.82 $\pm$ 0.07	0.82 $\pm$ 0.07	0.82 $\pm$ 0.07	0.86 $\pm$ 0.05	0.86 $\pm$ 0.05	0.85 $\pm$ 0.07	0.81 $\pm$ 0.03	0.79 $\pm$ 0.04	0.83 $\pm$ 0.03	0.68 $\pm$ 0.05	0.73 $\pm$ 0.05	0.83 $\pm$ 0.03	0.68 $\pm$ 0.05
LVPWs	0.98 $\pm$ 0.11	1.05 $\pm$ 0.07	0.97 $\pm$ 0.05	0.97 $\pm$ 0.05	1.07 $\pm$ 0.05	1.07 $\pm$ 0.05	1.08 $\pm$ 0.09	1.14 $\pm$ 0.08	0.96 $\pm$ 0.04	1.06 $\pm$ 0.06	0.86 $\pm$ 0.08	0.89 $\pm$ 0.08	1.06 $\pm$ 0.06	0.86 $\pm$ 0.08
LVEDV	75.4 $\pm$ 5.8	60.6 $\pm$ 4.23	83.6 $\pm$ 4.4	83.6 $\pm$ 4.4	70.2 $\pm$ 4.6	70.2 $\pm$ 4.6	99.3 $\pm$ 7.1	85.5 $\pm$ 6.2	97.8 $\pm$ 6.1	96.9 $\pm$ 9.7	106.2 $\pm$ 11.9	98.7 $\pm$ 7.3	96.9 $\pm$ 9.7	106.2 $\pm$ 11.9
LVESV	34.0 $\pm$ 4.4	23.31 $\pm$ 2.1	50.3 $\pm$ 4.6	50.3 $\pm$ 4.6	37.4 $\pm$ 4.1	37.4 $\pm$ 4.1	58.6 $\pm$ 7.7	45.2 $\pm$ 6.8	64.8 $\pm$ 7.1	56.9 $\pm$ 10.6	71.4 $\pm$ 14.1	63.1 $\pm$ 7.6	56.9 $\pm$ 10.6	71.4 $\pm$ 14.1
LV mass	109 $\pm$ 6.1	108 $\pm$ 8.7	150 $\pm$ 12.1	150 $\pm$ 12.1	134 $\pm$ 9.6	134 $\pm$ 9.6	147 $\pm$ 11.9	152 $\pm$ 14.2	161 $\pm$ 13.1	149 $\pm$ 7.2	151 $\pm$ 9.1	163 $\pm$ 10.7	149 $\pm$ 7.2	151 $\pm$ 9.1
HR indicates heart rate; IVSd, interventricular septal thickness at diastole; IVSs, interventricular septal thickness at systole; LVPWd, left ventricular posterior wall thickness at diastole; LVPWs, left ventricular posterior wall thickness at systole; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume.														

CHAPTER 6: DISCUSSION

6.1 Introduction

My dissertation addresses new functions for the E3 ubiquitin ligase Parkin in cells. Parkin is expressed in most tissues throughout the body, and has substrates involved in a variety of cellular processes. Loss of function mutations in Parkin can cause PD, and can affect its ligase function, subcellular localization, or cause protein misfolding and aggregation. Mice lacking Parkin altogether are more sensitive to cardiac stressors and are at an increased risk of developing cancer. Understanding Parkin's function in health and disease has been a primary focus of recent research.

Parkin's role at the mitochondria has been well-characterized, but its functions in other subcellular locations are less well-defined. Additionally, the cellular conditions that cause Parkin translocation to and from other cellular compartments are incompletely understood. In this dissertation, I have identified three distinct cellular stressors that affect Parkin's localization. During ER-stress, Parkin translocates to the ER. Parkin also translocates to the nucleus in response to hypoxia, but rapidly exits the nucleus in response to nutrient deprivation. Further study of Parkin's localization in response to additional cellular stressors will be necessary for identifying novel substrates and functions.

Parkin has several validated mitochondrial substrates, but accumulating evidence suggests that additional, non-mitochondrial substrates exist that have yet to be identified. Instead of focusing on a single target and the functional consequences of its ubiquitination by Parkin, some studies have examined global effects of Parkin

through proteomics and transcriptomics (Planken et al., 2017; Sarraf et al., 2013; Shamir et al., 2017). My research establishes the transcriptional effects of Parkin's nuclear localization. Furthermore, I identify a transcription factor substrate of Parkin in the nucleus.

Parkin-mediated mitophagy is protective in the heart, and loss of Parkin in mice increases susceptibility to cardiac injury. However, whether increasing Parkin protein levels is beneficial during chronic heart disease was unclear. My studies investigated whether Parkin overexpression altered mitochondrial clearance and heart function in a model of chronic cardiac pressure-overload. While increasing Parkin did not change mitochondrial content, cardiac hypertrophy, or cardiac function, my findings suggest that hearts from Parkin TG mice may have altered mitochondrial function in response to TAC.

The research presented in this dissertation therefore expands upon our current knowledge of Parkin's subcellular localization, function, and disease relevance. I demonstrate that Parkin translocates to various cellular compartments in a stress-dependent manner. I also identify transcriptional effects of nuclear-localized Parkin, and characterize a nuclear substrate of Parkin. Finally, I show that increasing Parkin in the heart does not alter the functional progression of pressure-overload-induced heart disease.

6.2 Parkin Localizes to Multiple Cellular Locations in a Stress-Dependent Manner

For a long time, it was believed that Parkin localizes to the cytosol basally, where it remains inactive until recruited to the outer mitochondrial membrane upon

mitochondrial depolarization. One of the more striking findings in this study was Parkin's recruitment to various cellular compartments in a stress-dependent manner. At baseline, Parkin localizes throughout the cell, not solely in the cytosol. Additionally, I found that Parkin translocates to the ER during ER stress. Although a portion of Parkin localizes to the nucleus basally, during nutrient deprivation, Parkin rapidly exits the nucleus. Conversely, during hypoxia, Parkin translocates to the nucleus. Parkin's translocation to damaged mitochondria is dependent upon phosphorylation by kinase PINK1. Its nuclear translocation, however, occurs independently of PINK1.

Interestingly, a recent study has shown that Parkin can be activated independently of PINK1 by an alternate kinase, AMPK (Lee et al., 2019). Furthermore, AMPK-dependent activation of Parkin is both cellular-stress-dependent and has effects separate to that of PINK1-dependent activation. AMPK is activated when cellular levels of ATP are low, such as during glucose deprivation. It is possible that AMPK-mediated phosphorylation affects Parkin's nuclear localization. However, as both nutrient deprivation and hypoxia lower cellular ATP levels, so it is also possible that these processes are regulated through an additional, currently unidentified mechanism.

6.3 Parkin Regulates Transcription of Metabolic Genes from the Nucleus

In this dissertation, I examine consequences of Parkin's nuclear localization. To do this, I generated a nuclear-targeted Parkin construct, NLS-Parkin. Importantly, NLS-Parkin localizes to the nucleus both at baseline and after mitochondrial damage, when WT Parkin robustly translocates to the outer mitochondrial membrane. Although it remains in the nucleus, NLS-Parkin still promotes mitochondrial clearance in cells

treated with various mitochondrial uncoupling agents. This functional consequence may be due to increased transcription of mitophagy receptor FUNDC1 in NLS-Parkin-expressing cells. Conversely, NLS-Parkin does not promote mitophagy in response to hypoxia. These data further indicate that Parkin acts as a stress-sensing protein and Parkin-mediated ubiquitination is dependent upon cellular context.

During normoxia, WT Parkin does not affect the transcriptome. However, NLS-Parkin expression alters transcript levels of 168 genes, around 70% of which are downregulated. Amino acid and ketone metabolism are among the top biological pathways implicated by NLS-Parkin-mediated gene expression. Among the potential upstream regulators of the metabolism-related transcripts were multiple nuclear receptors and co-activators. Collectively, these data indicate that, although WT Parkin does not affect the transcriptome at baseline, targeting Parkin to the nucleus affects basal metabolic gene transcription.

In contrast to normoxic conditions, during hypoxia, WT Parkin expression promotes the differential expression of 158 genes. Of these, 58 directly overlapped with genes differentially expressed with NLS-Parkin expression during hypoxia. Pathway analysis identified enrichment of these genes in HIF-1 signaling, the heat shock protein response, as well as metabolic processes. Similar to targeting Parkin to the nucleus under normoxic conditions, upstream regulators of genes differentially expressed in response to WT Parkin and NLS-Parkin during hypoxia also included several nuclear receptors known to regulate metabolic gene expression. Importantly, this similarity highlights how targeting Parkin to the nucleus produces a similar effect to stimulating Parkin nuclear translocation via hypoxia. Taken together, these data demonstrate a

substantial role for nuclear Parkin in regulating transcription of genes involved in metabolic processes. Interestingly, Parkin has previously been implicated in metabolic processes, though not specifically through functions in the nucleus. For example, Parkin-null mice are resistant to high-fat diet-induced weight gain. (Kim et al., 2011). Additionally, transcriptional studies have noted metabolic changes in the blood of patients with PD (Shamir et al., 2017). One mechanism for Parkin's effect on metabolism is through ubiquitination and stabilization of fatty acid translocase CD36 (Kim et al., 2011). However, additional mechanisms likely exist. The data presented here suggest that Parkin-mediated metabolic changes may occur through its presence in the nucleus.

Finally, I identified $ERR\alpha$ as a potential substrate of nuclear-localized Parkin. Nuclear-targeted Parkin interacts with $ERR\alpha$, and expression of NLS-Parkin increases transcription of $ERR\alpha$ targets. $ERR\alpha$ is a transcription factor that affects a wide-range of cellular processes including cellular fatty acid import and oxidation, mitochondrial respiratory function, glucose and glycogen metabolism, cholesterol metabolism, and gene regulation. Interestingly, similar to Parkin, $ERR\alpha$ is highly expressed in tissues that use fatty acids for energy. Additionally, $ERR\alpha$ has been implicated in both heart disease and cancer (Ariazi et al., 2002; Dufour et al., 2007; Huss et al., 2007; Ranhotra, 2015). A recent study has elucidated a mechanism for AMPK-activated $ERR\alpha$ regulation of autophagy (Kim et al., 2018). Given the functional overlap between $ERR\alpha$ and Parkin, additional studies should focus on how these proteins interact during disease states and whether their interaction may be targeted for potential therapies.

6.4 Parkin Expression does not Rescue TAC-induced Heart Disease

Although loss of Parkin increases susceptibility to both acute and chronic cardiac stress in mice, it was previously unknown whether increasing Parkin expression would be beneficial or detrimental. I found that overexpressing Parkin in the heart did not affect mitochondrial content in mice at baseline and in response to cardiac pressure-overload. Hearts from Parkin TG mice, did, however exhibit increased Complex II-dependent respiration and elevated mRNA levels of $ERR\alpha$ at two-weeks post-TAC, suggesting metabolic alterations. Although transcript levels of $ERR\alpha$ were increased, there was no corresponding increase on the transcriptional targets that I examined. Therefore, either $ERR\alpha$ was increased, but not active, or its activity affected other genes that were not tested. Parkin TG mice also developed perivascular fibrosis at a slower rate than WT mice in response to TAC. However, overexpressing Parkin in the heart did not affect TAC-induced decline in cardiac function or development of cardiac hypertrophy.

One of the primary goals of this study was to determine whether Parkin overexpression would be beneficial in response to chronic cardiac pressure-overload. Previous research has shown that deleting Drp1 from adult mouse hearts upregulates Parkin (Song et al., 2015). This increase in Parkin caused excessive mitophagy that contributed to lethal cardiomyopathy. With increased Parkin expression, there is the risk of depleting mitochondria through Parkin-mediated mitophagy. In this study, mitophagy was likely activated by deletion of Drp1. However, my results show that during heart failure, when there is less acute damage of mitochondria, increasing Parkin expression

does not cause excessive mitophagy, therefore allowing for increased Parkin to remain a potential therapeutic target for future work to continue to characterize.

6.5 Model

In light of my findings, I propose a model that expands upon the currently established views of Parkin localization and functions. Canonically, Parkin resides in the cytosol under baseline conditions, and translocates to the mitochondria upon mitochondrial depolarization (Figure 6.1 A). The data presented here provide additional contexts in which Parkin translocates to other cellular compartments. During ER stress brought on by disrupting calcium homeostasis, Parkin translocates to the ER (Figure 6.1 B). Parkin rapidly exits the nucleus in response to nutrient deprivation (Figure 6.1 C), but translocates to the nucleus during hypoxia (Figure 6.1 D). Thus, Parkin's subcellular localization is dynamic and stress-dependent.

Additionally, Parkin canonically functions to clear damaged mitochondria by ubiquitinating proteins on the outer mitochondrial membrane. My studies have shown several new functions of Parkin in the nucleus. During mitochondrial depolarization, nuclear Parkin can facilitate mitochondrial clearance, potentially through increasing transcription of mitophagy receptors. During hypoxia Parkin promotes transcriptional changes to genes that regulate metabolism from the nucleus (Figure 6.2). Finally, my data suggest that nuclear Parkin interacts with ERR α and promotes transcription of ERR α targets (Figure 6.3).

6.6 Future Studies

The research in this study supplements our current understanding of Parkin function. However, these studies have generated several unanswered questions that should be addressed in future work. Much of the research regarding Parkin has been conducted using pharmacological uncouplers that cause extensive mitochondrial depolarization and robust translocation of Parkin to the mitochondria. However, when a mitochondrion becomes damaged, it sequesters dysfunctional proteins into one location and divides asymmetrically to maintain protein quality and polarization (Twig et al., 2008). Therefore, physiologically, it is more likely that few mitochondria are depolarized, and rarely the entire mitochondrial network. When fewer mitochondria are damaged, it may allow the remaining Parkin to be recruited to other cellular compartments. My findings in Chapter 4 indicate that nuclear-localized Parkin basally promotes increased transcription of genes that regulate mitochondrial biogenesis, such as $ERR\alpha$, and $PGC1\alpha$. Therefore, it is possible that nuclear and mitochondrial Parkin facilitate mitochondrial clearance, while nuclear Parkin also promotes mitochondrial biogenesis to replenish the depleted pool of functional mitochondria. Future work should address whether nuclear-localized Parkin increases mitochondrial turnover, and how it effects mitochondrial function.

I was unable to identify the presence of a NLS in Parkin (Chapter 2), thus it is still unclear how Parkin's nuclear localization is altered. Parkin is regulated by post-translational modifications (Chakraborty et al., 2017). Beyond altering Parkin activity, post-translational modifications also affect its subcellular localization. In response to mitochondrial depolarization, PINK1 phosphorylates Parkin promote its translocation to the mitochondria (Kim et al., 2008; Kondapalli et al., 2012; Sha et al., 2010).

Additionally, S-nitrosylation of Parkin sequesters Parkin to the cytosol (Sunico et al., 2013). Therefore, it is possible that a post-translational modification promotes Parkin's nuclear localization. Future studies should examine how post-translational modifications alter Parkin's localization and whether Parkin is modified differently in separate subcellular compartments.

Additionally, although I chose to focus my research on consequences of Parkin in the nucleus, in Chapter 2 I found that Parkin rapidly exits the nucleus in response to nutrient deprivation. Whether the main consequence is loss of Parkin's basal nuclear function, increasing its activity in the cytoplasm, or both is unknown. Therefore, additional research should focus on Parkin's functions in response to nutrient deprivation. One interesting avenue to explore is whether Parkin has an endogenous nuclear export signal. Exposure of an endogenous nuclear export signal is one potential mechanism for Parkin's rapid exit from the nucleus in response to nutrient deprivation. Additionally, a recent study has shown that AMPK phosphorylates Parkin to increase its ubiquitination of RIPK3 (Lee et al., 2019). As AMPK is activated when cellular levels of ATP are low, AMPK-mediated phosphorylation of Parkin may occur during nutrient deprivation and promote Parkin's translocation from the nucleus.

One limitation of the experiments presented here is the use of immortalized HeLa cells and MEFs. Although I confirmed that Parkin localizes to the nucleus basally in various tissues in mice, I did not examine whether nutrient deprivation or hypoxic conditions alters Parkin's subcellular localization. Future studies may examine whether fasting or oxygen deprivation in mice alters Parkin's subcellular localization, especially in neurons and cardiac myocytes. Additionally, although my research revealed changes

in metabolic gene transcription, I did not assess the functional consequences. Therefore, future studies may determine whether hypoxia-mediated Parkin nuclear localization increases lipid uptake or diet-induced weight gain in mice.

6.7 Relevance to Disease and Therapeutic Potential

While loss of function of Parkin has been linked to PD, cardiovascular disease, and cancer, the mechanisms remain unclear. Similarly, while several substrates of Parkin have been identified and validated, how their ubiquitination contributes to cellular homeostasis and health is incompletely understood.

Here, I've shown that Parkin has several functions in the nucleus, but that PD-associated mutants of Parkin are excluded from the nucleus. This work reveals that loss of Parkin's nuclear roles may contribute to development of PD. Nuclear Parkin facilitates mitophagy in response to mitochondrial stress and alters transcription of genes that regulate metabolism both basally and in response to hypoxia. Future studies should determine whether PD-patients with mutations in *PRKN* experience dysregulation of pathways associated with Parkin's functions in the nucleus.

During an MI, the myocardium is deprived of both oxygen and other nutrients. Parkin levels are increased at the mitochondria in the border zone after an MI (Kubli et al., 2013b), likely to promote clearance of damaged mitochondria. However, it is unclear whether Parkin is also enriched in other cellular compartments. It is possible that lacking Parkin in either the nucleus or the cytosol contributes to the sensitivity of Parkin-null mice to MI.

Parkin levels are often increased during various cellular stressors. However, whether they are increased similarly across cellular compartments or enriched in specific locations is unknown. My research suggests that Parkin may have complementary functions in different cellular compartments. For example, Parkin labels the mitochondria for clearance during mitochondrial depolarization, but may promote both mitophagy and mitochondrial biogenesis from the nucleus.

Understanding localization-specific functions of Parkin may expose additional substrates and signaling pathways, thereby increasing our understanding of Parkin's functions and allowing for the generation of treatments with fewer inadvertent consequences.

6.8 Concluding Remarks

Although Parkin was originally identified for its relevance to PD, accumulating evidence indicates that it is also implicated in cardiovascular disease and cancer. The work presented here expands our knowledge of Parkin localization and function in cells. Specifically, it highlights the notion that Parkin is a cellular stress-sensing protein and provides specific physiologic contexts for its actions in the cell. It additionally characterizes roles for Parkin in the nucleus at baseline and in response to mitochondrial depolarization and hypoxia. A complete understanding of how cellular stressors activate Parkin's various functions is a crucial step towards being able to therapeutically target Parkin.

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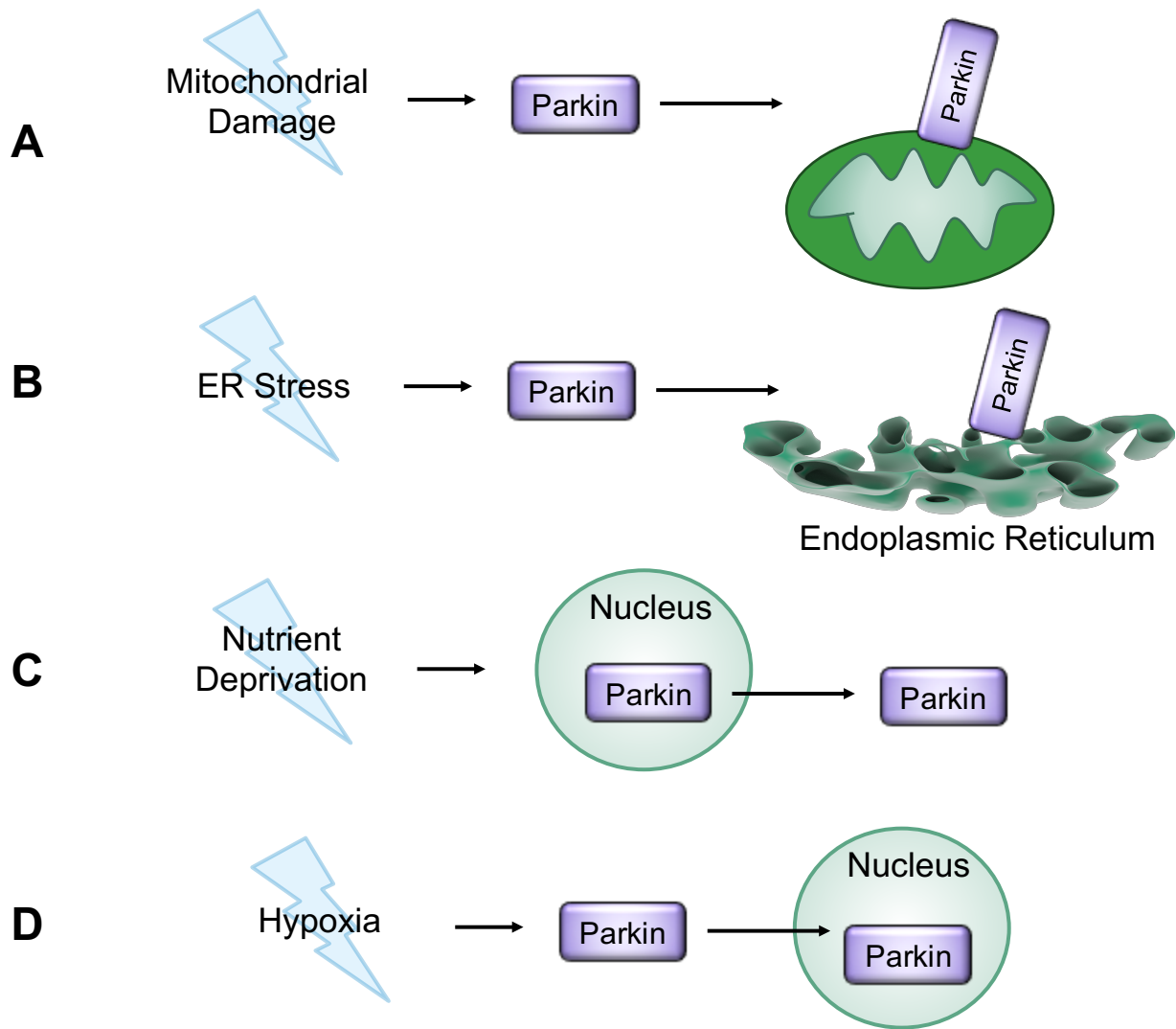


Figure 6.1. Parkin's Subcellular Localization is Dynamic and Stress-Dependent. A) Upon mitochondrial damage, Parkin translocates to the damaged mitochondria. B) My data presented here show that Parkin translocates to endoplasmic reticulum (ER) during ER stress. C) During nutrient deprivation, Parkin rapidly exits the nucleus. D) Parkin translocates to the nucleus in response to hypoxia.

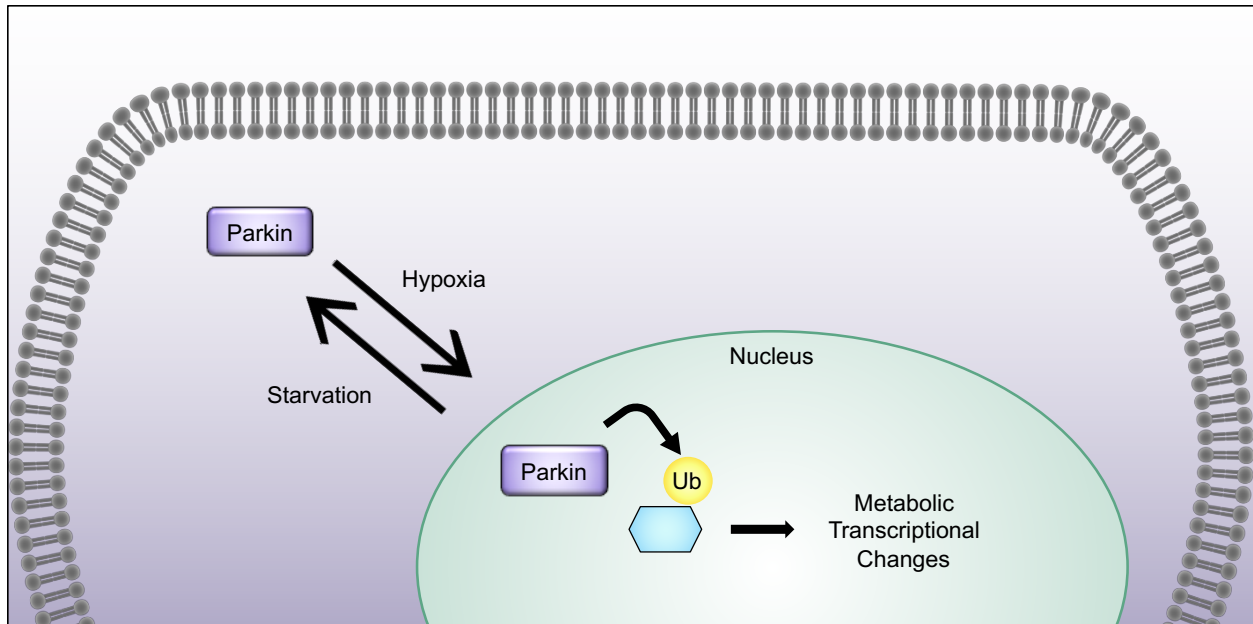


Figure 6.2 Model of Parkin Nuclear Localization and its Effect on the Transcriptome. Parkin exits the nucleus in response to nutrient deprivation, but translocates to the nucleus during hypoxia. Parkin ubiquitinates nuclear proteins including transcription factors, which causes transcriptional changes to several metabolic genes.

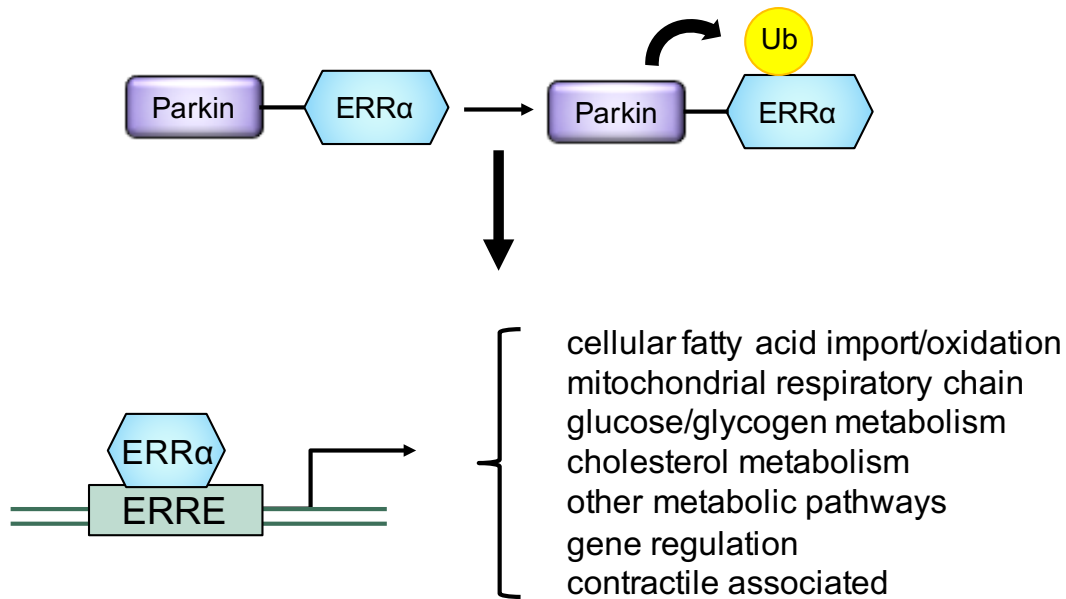


Figure 6.3 Proposed mechanism of Parkin-Mediated ERRα Target Transcription. Parkin interacts with and ubiquitinates transcription factor ERRα. Parkin-mediated ubiquitination of ERRα increases its activity to promote transcriptional changes to genes responsible for regulating several pathways including mitochondrial biogenesis, metabolism, and gene regulation.

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