The Role of Parkin and Mitophagy in Acetaminophen and Alcohol-induced Liver Injuries

By

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Submitted to the graduate degree program in Pharmacology, Toxicology, and Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The Role of Parkin and Mitophagy in Acetaminophen and Alcohol-induced Liver Injuries

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Date approved: June 8th, 2015

Abstract

Acetaminophen (APAP) is the leading cause of acute liver failure in the United States, and alcoholic liver disease (ALD) is a worldwide health problem that claims two million lives per year. Currently, the only cure for either disease is liver transplantation in severe disease states. Therefore, new therapeutic options for treatment of these liver diseases are greatly needed. To develop new therapeutic options, the mechanisms involved in APAP and alcohol-induced liver toxicities must be better understood. We previously demonstrated that autophagy was protective against both APAP and alcohol-induced liver injuries by removing damaged mitochondria by mitophagy, which is a selective form of autophagy specific for mitochondria. However, the mechanisms for induction of mitophagy in the liver are unknown. Parkin is an E3 ubiquitin ligase that is well known to be required for mitophagy induction in mammalian cell models after mitochondrial depolarization. Therefore, we evaluated the role of Parkin in inducing mitophagy as a protective mechanism against APAP and alcohol-induced liver injuries. For alcohol treatment, acute-binge and chronic-plus-binge (Gao-binge) models were used.

First, we found that APAP and alcohol produced opposite responses in Parkin KO mice. Parkin KO mice were protected after APAP treatment, but they had more liver injury and steatosis after alcohol treatment compared to WT mice. It is well known that c-Jun N-terminal kinase (JNK) activation exacerbates APAP-induced liver injury, and it has recently been shown that myeloid leukemia cell differentiation protein (Mcl-1) mediates protection against APAP-induced liver injury. In addition, liver regeneration has been shown to be the most important repair mechanism for APAP-induced liver injury. We found that Parkin KO mice had decreased JNK activation, increased Mcl-1 expression, and increased hepatocyte proliferation after APAP treatment in their livers compared to WT mice. In contrast to protection after APAP treatment, Parkin KO mice were more susceptible to alcohol-induced liver injury than WT mice because of Parkin's role in maintaining a healthy population of mitochondria, likely through activation of

mitophagy. Alcohol caused greater mitochondrial damage in Parkin KO livers compared to WT livers. Parkin KO mice had severely swollen and damaged mitochondria that lacked cristae after alcohol treatment, which were not seen in WT mice. In addition, Parkin KO mice had decreased mitophagy, β-oxidation, mitochondrial respiration, and cytochrome c oxidase activity after acute-binge alcohol treatment compared to WT mice. Furthermore, Parkin KO mouse liver mitochondria had less capacity to adapt to Gao-binge treatment compared to WT mouse liver mitochondria. The fact that Parkin KO mice were protected against APAP-induced liver injury but had increased liver injury and steatosis after alcohol treatment compared to WT mice suggests that Parkin has multiple roles in maintaining cellular homeostasis in addition to its role in initiation of mitophagy, and these various roles my differ in importance depending on the amount and type of liver injury produced. Therefore, Parkin's roles in regulating proliferation, JNK activation, and Mcl-1 expression were likely more important than mitophagy during APAP-induced liver injury while its role in mitophagy induction to maintain mitochondrial homeostasis was likely more important during alcohol-induced liver injury.

Second, we surprisingly found that even though Parkin has been shown to be required for mitophagy induction in *in vitro* models, Parkin was not essential for mitophagy induction in the liver. We found that mitophagy still occurred in Parkin KO mice based on electron microscopy analysis after APAP and alcohol treatments. However, mitophagy levels were reduced in Parkin KO mice compared to WT mice, and Parkin translocated to mitochondria in WT mouse livers after both APAP and alcohol treatments. These results suggest that Parkin-induced mitophagy is still likely an important protective mechanism in the liver even though compensatory adaptive mechanisms exist for mitophagy induction in the absence of Parkin. In addition, these compensatory mechanisms may not be as efficient as Parkin in inducing mitophagy in the liver because mitophagy levels were reduced in Parkin KO mice compared to WT mice after APAP and alcohol treatments. Parkin-independent mechanisms for mitophagy

induction in the liver are currently unknown, but they may involve activation of other proteins known to mediate mitophagy, such as Mul1, which has been shown to act in parallel to the Parkin pathway in *Drosophila*.

Third, we found that whole-body knockout of Parkin and acute knockdown of Parkin had opposite responses to APAP overdose. While Parkin KO mice were protected against APAP-induced liver injury compared to WT mice, acute knockdown of Parkin in mouse livers resulted in increased liver injury compared to WT mice after APAP treatment. Whole-body Parkin KO mice were protected because they had increased Mcl-1 and proliferation levels and decreased JNK activation, and mice with acute knockdown had exacerbated APAP-induced liver injury because they had reduced Mcl-1 and proliferation levels and increased JNK activation. In addition, mitophagy was reduced in both Parkin KO mice and mice with acute knockdown of Parkin compared to WT mice. However, mitophagy levels were only slightly reduced in whole-body knockout mice while they were significantly reduced in mice with acute Parkin knockdown. These opposite responses between Parkin whole-body KO and acute knockdown mice were likely due to a lack of time to develop compensatory and adaptive mechanisms in the acute knockdown mice that were present in the whole-body knockout mice.

Overall, our findings indicate that Parkin-induced mitophagy is likely a mechanism of protection in the liver, but compensatory mechanisms exist for induction of mitophagy in the absence of Parkin. However, these compensatory mechanisms may only play a minor role in the liver. In addition, Parkin has multiple roles in maintaining cellular homeostasis in addition to mitophagy, which causes Parkin KO mice to respond differently to various types of liver injury. However, our findings from Parkin KO mice likely better reflect physiological conditions in people that have chronic loss of Parkin function, such as Autosomal-Recessive Parkinson's disease patients, who have mutations in the Park2 (Parkin) gene. Finally, our results indicate that evaluation of drug-induced liver injury mechanisms using whole-body knockout mice should

be interpreted with caution due to adaptive and compensatory mechanisms that may be activated in knockout mice. In addition, modulating Parkin-mediated mitophagy may be a promising therapeutic approach for targeting drug and alcohol-induced liver injuries.

Acknowledgments

Thank you to my wonderful husband, David. Without your encouragement and support, I never would have entered (or made it through) graduate school. You are my rock in life, and I am so lucky to have met you eleven years ago. You are an amazing husband and father, and I grow prouder of you and love you more each day.

Thank you to my beautiful daughter, Hailey Grace, for giving me more joy and love in life than I ever thought possible. You inspire me with your constant laughter and curiosity, and you know how to keep me on my toes. I cannot wait to see what your future holds.

Thank you to my parents, Richard and Charlotte Franklin, for your continuous support and love. Thank you to my mother for being a friend to me, as well as a parent. And to my father, for encouraging me to always do my best. Thank you also to both of my parents for constantly reminding me about the importance of education. I would not be where I am today without your guidance.

Thank you to my baby brother, Justin Franklin, for always making me smile and for giving great bear hugs. It has been a joy watching you grow up, and I am so proud of you.

Thank you to my mother and father in-law, Ann and Will, and to my sister-in-law, Jennifer, for accepting me into your loving family and also for your never-ending support. I am blessed to be a part of your lives. Thank you also to Ann and Will for caring for Hailey while I finished my doctorate.

Thank you Dr. Wen-Xing Ding for accepting me into your lab and for always pushing me to be the best scientist I can be. I have grown not only as a scientist, but also as a person by working with you. I could not have asked for better guidance, or to be taught by a more passionate scientist, during my doctoral training. Thank you also for your concern and generosity when I needed time away to care for my husband or my daughter. I have learned so much from you, and I am blessed to have you as my mentor.

Thank you to members of the Ding lab for always providing a happy place to do science. I consider you all my “science family”, and I have very much enjoyed working with and learning from each of you. Thank you to Hongmin Ni for always helping me in the lab and for being a great friend. I have learned a lot from you, and I truly appreciate everything you have done for me. Thank you also to Sharon Manley for your friendship, great humor, and for teaching me sign language! Yuan Li, Shaogui Wang, Xiao Yang, Xiaojuan Chao, Yan Zhou, Hua Yang, and Anna Haynes, working with you has been an experience I will never forget! Thank you all for your support and friendship.

Thank you to my mentor Dr. Grace Guo for taking me under your wing when I first entered graduate school. I learned so much from you, and I am very grateful to have spent two years as your student. Thank you also to members of the Guo lab for your friendship and help in the lab. Particularly, thank you to Dr. Bo Kong for always making me smile and laugh and for feeding me yummy Chinese meals. I am so glad that our friendship has continued even though we no longer work together.

Thank you to my dissertation committee Dr. Hartmut Jaeschke, Dr. Michele Pritchard, Dr. Udayan Apte, and Dr. Benyi Li for your advice and guidance, and for helping making my projects in the lab better.

Thank you to Olivia Christie, Sarah Peterson, and Megan Ford for supporting me through almost every milestone in life so far. Friends like you do not come along every day, and I am truly blessed to have each of you in my life. Thank you for your comic relief, chats, and bottomless wine glasses.

Thank you Dr. Greg Reed (“Uncle Greg”) for your encouragement, guidance, and good humor. I have enjoyed being your “niece”.

Thank you Barbara Fegley for your help with electron microscopy studies, Dr. Russell Swerdlow and Dr. Heather Wilkins for your assistance with mitochondrial respiration analysis,

and Margitta Lebofsky for your help with glutathione measurement. Also, thank you to Cody Tully, Dorothy McGregor, and Rosa Meagher for your amazing organization skills and friendship.

Finally, thank you to my furry babies, Emma and Lily, for providing cuddles and kisses.

Dedicated to:

Hailey Grace Williams

You made my dreams come true.

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Chapter 1. Introduction

1.1 Autophagy-Lysosome and Ubiquitin-Proteasome Systems for Protein Degradation

Intracellular protein degradation is a necessary process for maintaining cellular function and health, and it occurs via two unique systems: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system. The UPS is important for degradation of approximately 80% of proteins including short-lived or abnormally folded and damaged proteins. Many of these proteins are involved in processes including DNA repair, transcription, translation, cell cycle, and cell stress response (Lilienbaum 2013). The proteasome contains both a core 20S subunit and a 19S regulatory subunit. The 20S subunit contains proteases and peptidases capable of breaking down proteins into single amino acids. The 19S subunit helps the 20S subunit recognize ubiquitinated proteins while also unfolding and translocating them to the 20S subunit. In addition to regulation by the 19S subunit, the 26S proteasome can also be regulated post-translationally (Schmidt and Finley 2014). The autophagy-lysosome system is an evolutionary conserved pathway that is necessary for degradation of long-lived proteins and organelles in addition to aggregated proteins (Korolchuk, Menzies et al. 2010, Lilienbaum 2013). Autophagy is extensively discussed in section 1.2. Both of these systems help during starvation conditions by providing recycled amino acids, and they also both protect against cellular dysfunction and death by preventing the accumulation of damaged proteins.

In order for proteins to be degraded by the UPS, they must first be covalently tagged with ubiquitin, which occurs through a series of steps involving E1, E2, E3, and E4 ubiquitin ligases and other enzymes. Ubiquitin attaches to the E1 activating enzyme by binding its carboxy-terminal glycine to one of the E1 enzyme's cysteine residues, which requires ATP. The activated ubiquitin is then transferred to a cysteine residue of an E2 conjugating enzyme. In the third step, the ubiquitin is attached to a protein that is already bound by an E3 ligase (Kornitzer and Ciechanover 2000). This can occur by two methods depending on if the E3 ligase contains a RING (really interesting new gene) domain or HECT (homologous to the E6AP carboxy

terminus) domain. In the case of the E3 ligase containing a RING domain, the E3 ligase transfers ubiquitin directly to the targeted protein (Metzger, Pruneda et al. 2014). When an E3 ligase contains a HECT domain, ubiquitin is first transferred to a catalytic cysteine residue within the HECT domain, then the E3 ligase transfers the ubiquitin to the targeted protein (Scheffner and Kumar 2014). Once the first ubiquitin is attached, the targeted protein can become polyubiquitinated by attachment of additional ubiquitin proteins. The process of polyubiquitination can utilize an E4 ubiquitin ligase / polyubiquitinating enzyme, which attaches multiple ubiquitins to a lysine residue on an already protein-bound ubiquitin and signals for the protein to be degraded by the proteasome (Koegl, Hoppe et al. 1999, Lilienbaum 2013) (**Figure 1.1**). Ubiquitinated proteins can also be degraded by autophagy.

The autophagy and ubiquitin-proteasome systems must both be tightly regulated in order to maintain cellular homeostasis and survival, and they are each activated by different conditions in the cell. Generally, autophagy degrades long-lived proteins (Yorimitsu and Klionsky 2005) while the UPS degrades short-lived proteins (Goldberg 2003). In addition, autophagy can degrade protein aggregates, while the UPS is restricted to degradation of soluble proteins (Korolchuk, Menzies et al. 2010, Lamark and Johansen 2012). The UPS and autophagy are also activated by ubiquitin-tagging of different lysine residues. The UPS is generally activated by polyubiquitination (at least 4 ubiquitins) of ubiquitin lysine 48 residues (Baboshina and Haas 1996, Thrower, Hoffman et al. 2000), while autophagy is generally activated by polyubiquitination of ubiquitin lysine 63 residues (Tan, Wong et al. 2008). Even though each system is activated by different signals, autophagy can compensate for a defective UPS to degrade damaged proteins (Ding, Ni et al. 2007, Pandey, Batlevi et al. 2007, Korolchuk, Menzies et al. 2010). However, the UPS cannot compensate for defective autophagy (Korolchuk, Mansilla et al. 2009, Korolchuk, Menzies et al. 2010).

Figure 1.1

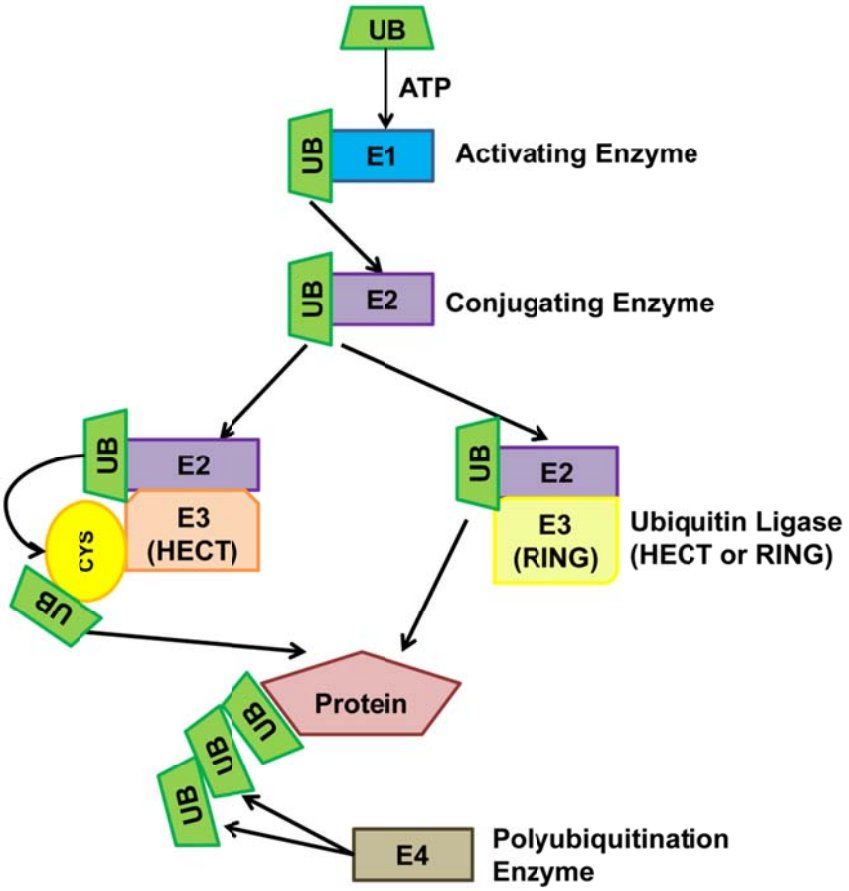

Figure 1.1: Ubiquitination of target proteins for degradation by the proteasome or lysosome requires E1, E2, E3, and E4 ligases.

1.2 Autophagy

1.2.1 Three Types of Autophagy

Autophagy occurs via three distinct pathways in mammalian cells: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. All three pathways result in delivery of cargo to the lysosome for degradation.

Microautophagy is a non-selective process induced in starvation conditions where portions of cytoplasm and intact organelles are engulfed directly by lysosomes via lysosome membrane protrusion without prior sequestration into autophagosomes. The invaginated lysosome containing its cargo then extends into an autophagic tube and contents are degraded in the lysosome (Mijaljica, Prescott et al. 2011). Currently, little is known about the regulation of microautophagy or its functional role in human health.

Chaperone-Mediated Autophagy (CMA) only occurs in mammalian cells, and it is activated during prolonged starvation in order to provide nutrients and energy by degrading unnecessary proteins to replenish amino acids (Cuervo, Knecht et al. 1995). CMA is also activated during cellular injury, such as during oxidative stress for degradation of oxidized proteins (Kiffin, Christian et al. 2004). CMA is characterized by selection of specific cytosolic proteins by the chaperone heat shock-cognate protein of 70 kDa (hsc70), which shuttles unfolded proteins directly across the lysosome membrane (Chiang, Terlecky et al. 1989). Similar to microautophagy, CMA allows for degradation of proteins in lysosomes without prior sequestration into autophagic vesicles. However, CMA differs from microautophagy because CMA is a selective process that uses chaperones to target cargo for degradation. During CMA, individual proteins containing the KFERQ motif are selected for degradation (Dice 1990). Approximately 30% of proteins in the cytosol contain this motif (Chiang and Dice 1988). Proteins degraded by CMA include glycolytic enzymes, proteasome subunits, and transcription factors

among others (Arias and Cuervo 2011). Once delivered to the lysosome surface by hsc70, the selected protein interacts with the lysosome membrane protein lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo and Dice 1996). The protein then interacts with the lysosome form of hsc70 (lys-hsc70), which helps unfold the protein and later facilitates its translocation across the lysosome membrane for degradation (Chiang, Terlecky et al. 1989, Salvador, Aguado et al. 2000, Agarraberes and Dice 2001, Orenstein and Cuervo 2010). Maintenance of appropriate CMA levels is important because disruptions in CMA balance contribute to pathologies in neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease when CMA is reduced, and upregulation of cancer cell proliferation and survival when CMA is overactive (Cuervo and Wong 2014). However, the exact mechanisms for CMA regulation are not fully understood.

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that results in a cell's "self-eating" to degrade cellular proteins and organelles and is the most studied autophagic process. Autophagy is different from both microautophagy and CMA because sequestration of cargo in the cytosol occurs away from the lysosome membrane through the formation of autophagosomes. Autophagy is a protective process that provides the cell with nutrients in response to starvation to produce energy. During starvation conditions, degradation of cytoplasmic proteins and organelles by autophagy provides amino acids that can be used in gluconeogenesis or to produce ATP by entering the Krebs cycle (Rabinowitz and White 2010). In addition, degradation of lipids by autophagy produces free fatty acids that can be used to produce energy by β-oxidation in the mitochondria (Singh, Kaushik et al. 2009). In addition to providing energy during starvation, autophagy also breaks down damaged proteins and organelles in order to prevent cellular injury. Double-membrane autophagosomes engulf these proteins and organelles in the cytoplasm and then fuse with lysosomes to degrade their components via lysosome proteases (Parzych and Klionsky 2014). Autophagy can be both a

selective and non-selective process. Non-selective autophagy breaks down proteins and organelles during starvation in order to provide the cell with necessary nutrients. Selective autophagy removes damaged organelles and protein aggregates using specific receptors, and it can occur in both nutrient-rich and poor conditions. (Reggiori, Komatsu et al. 2012). Mitophagy, the focus of this dissertation, is selective degradation of mitochondria by autophagy. Mitophagy along with other forms of selective autophagy are further discussed in section 1.3.

Autophagy is well studied in both yeast and mammalian cells. There are 5 steps in the autophagy process known as 1: initiation and nucleation of the phagophore/isolation membrane, 2: elongation of the isolation membrane, 3: closure and maturation to form a double-membrane autophagosome, 4: fusion with lysosomes (or endosomes followed by lysosomes) to form the autolysosome, and 5: degradation of cargo in lysosomes by lysosomal hydrolases (**Figure 1.2.1A).** Autophagy initiation occurs at a single phagophore assembly site (PAS) in yeast and is regulated by the Atg1-Atg13-Atg17-Atg31-Atg29 kinase complex (Feng, He et al. 2014). In mammalian cells, autophagy initiation is different than in yeast because it occurs at multiple cytosolic sites instead of a single PAS, and it is regulated by the UNC-51-like kinase 1 (ULK1) complex containing ULK1 (Atg1 yeast homolog), ATG13, RB1-inducible coiled-coil 1 (FIP200), and Atg101 (Itakura and Mizushima 2010, Chen and Klionsky 2011, Parzych and Klionsky 2014). Upon activation, ULK1 can auto-phosphorylate itself and then phosphorylate ATG13 and FIP200 to initiate autophagosome biogenesis (Jung, Jun et al. 2009).

After induction, nucleation occurs in both yeast and mammalian cells to generate phosphatidylinositol 3-phosphate (PtdIns(3)P), which is a phospholipid required for trafficking of phospholipid-binding proteins to the autophagosome membrane. Nucleation is regulated by a class III phosphatidylinositol 3-kinase (PtdIns3K) complex containing the PtdIns3K VPS34 (Vps34 in yeast), p150 (Vps15 in yeast), Beclin1 (Atg6 in yeast), and either ATG14 or UVRAG (Feng, He et al. 2014, Parzych and Klionsky 2014). ATG14 and UVRAG both bind to Beclin1

mutually exclusively. ATG14 in this complex is important for autophagy induction, and UVRAG in this complex can participate in both autophagy and endocytosis (Feng, He et al. 2014).

After induction and nucleation, autophagosome membrane elongation in both yeast and mammalian cells requires two ubiquitin-like conjugation systems to form a phagophore. In ubiquitin systems, an E1 ligase is a ubiquitin activating enzyme, an E2 ligase is a ubiquitin conjugating enzyme, and an E3 ligase assists in transferring ubiquitin from an E2 ligase to a substrate, as previously discussed in section 1.1. The first ubiquitin-like system in autophagy includes formation of a complex from the autophagy-related genes (Atg genes) Atg12, Atg5, and Atg16 in yeast. This complex forms by irreversible attachment of Atg5 to Atg12 by the E1-activating enzyme Atg7 and the E2-conjugating enzyme Atg10 (Ohsumi 2001, Yorimitsu and Klionsky 2005). Atg16 then reversibly binds the Atg5-Atg12 complex (Ohsumi 2001, Kuma, Mizushima et al. 2002, Yorimitsu and Klionsky 2005). Orthologs for mammalian cells are ATG5, ATG7, ATG12, and ATG16L1 (Ohsumi 2001). The second ubiquitin-like system is the Atg8 system. In yeast, Atg8 is cleaved by Atg4 and a glycine residue is exposed at the C terminus of Atg8, making it available for binding to the E1-like activating enzyme Atg7. Atg8 is then activated by Atg7, which then transfers it to the E2-like conjugating enzyme Atg3. Atg3 in conjunction with the E3-like Atg5-Atg12-Atg16 complex then mediates lipidation of Atg8 through conjugation of its exposed glycine residue to phosphatidylethanolamine (PE) (Ichimura, Kirisako et al. 2000). Atg8 conjugation with PE is reversible because Atg8 can be cleaved after the glycine residue to separate it from PE by Atg4 (Kirisako, Ichimura et al. 2000). The Atg5-Atg12-Atg16 complex mediates elongation and curvature of the autophagosome, but it dissociates from the autophagosome membrane after formation. Unlike the Atg5-Atg12-Atg16 complex, the Atg8-PE complex remains on the autophagosome membrane after formation (Yorimitsu and Klionsky 2005). The elongation process in mammalian cells is similar to yeast with LC3 acting as a homolog of Atg8. The cleaved form of LC3 with the exposed glycine residue is known as

LC3-1, and the PE-conjugated form is known as LC3-II (Geng and Klionsky 2008). Atg9 in yeast (ATG9 in mammals) is important for phagophore membrane expansion. It is unique because it is the only membrane-spanning Atg protein, and it helps induce membrane expansion by shuttling between the autophagosome formation site and peripheral sites to recruit membrane (Young, Chan et al. 2006). Sources for autophagosome membrane recruitment required for membrane elongation are still highly debated, but they are hypothesized to include endoplasmic reticulum, Golgi, the plasma membrane, and mitochondria (Parzych and Klionsky 2014).

Once elongated, the phagophore matures and closes into a double-membrane autophagosome, but this process is still not completely understood. The autophagosome later transports the cargo to the endosome or lysosome in mammalian cells or to vacuoles in yeast. In mammalian cells, movement of the autophagosome to the lysosome requires microtubules (Monastyrska, Rieter et al. 2009). In mammals, autophagosome-lysosome fusion requires both the GTPase RAB7 and the lysosome membrane protein LAMP-2. (Tanaka, Guhde et al. 2000, Jager, Bucci et al. 2004). UVRAG activates RAB7 to promote the fusion of autophagosomes with lysosomes to form autolysosomes (Jager, Bucci et al. 2004). In yeast, fusion of the autophagosome with a vacuole releases an autophagic body into the lumen of the vacuole where it is degraded by vacuolar hydrolases. In both yeast and mammalian cells, the cargo within the autophagosome along with the autophagosome inner membrane are degraded in the vacuole or lysosome, respectively. The components resulting from the degradation are then exported into the cytoplasm for further use in protein synthesis and energy production (He and Klionsky 2009). In mammalian cells, autophagosomes can also first fuse with endosomes before later fusing with lysosomes (Berg, Fengsrud et al. 1998). Autophagy machinery is summarized in **Figure 1.2.1B.**

Figure 1.2.1A

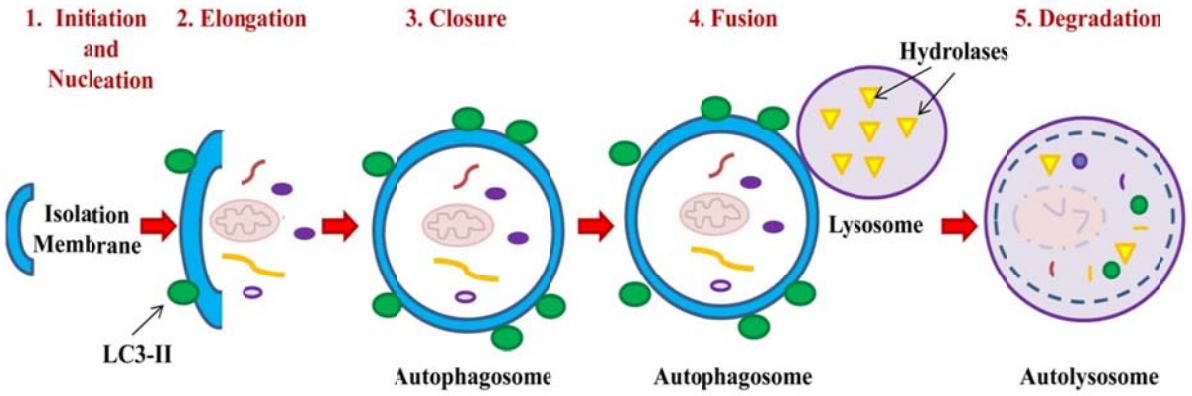

Figure 1.2.1A: Scheme of macroautophagy 1) Autophagy is initiated by the ULK1 complex. The membrane is then nucleated and 2) elongated before 3) closing into a double-membrane autophagosome, which contains cargo for degradation. 4) The autophagosome then fuses with the lysosome to form an autolysosome where 5) cargo is degraded by lysosome hydrolases.

Figure 1.2.1B

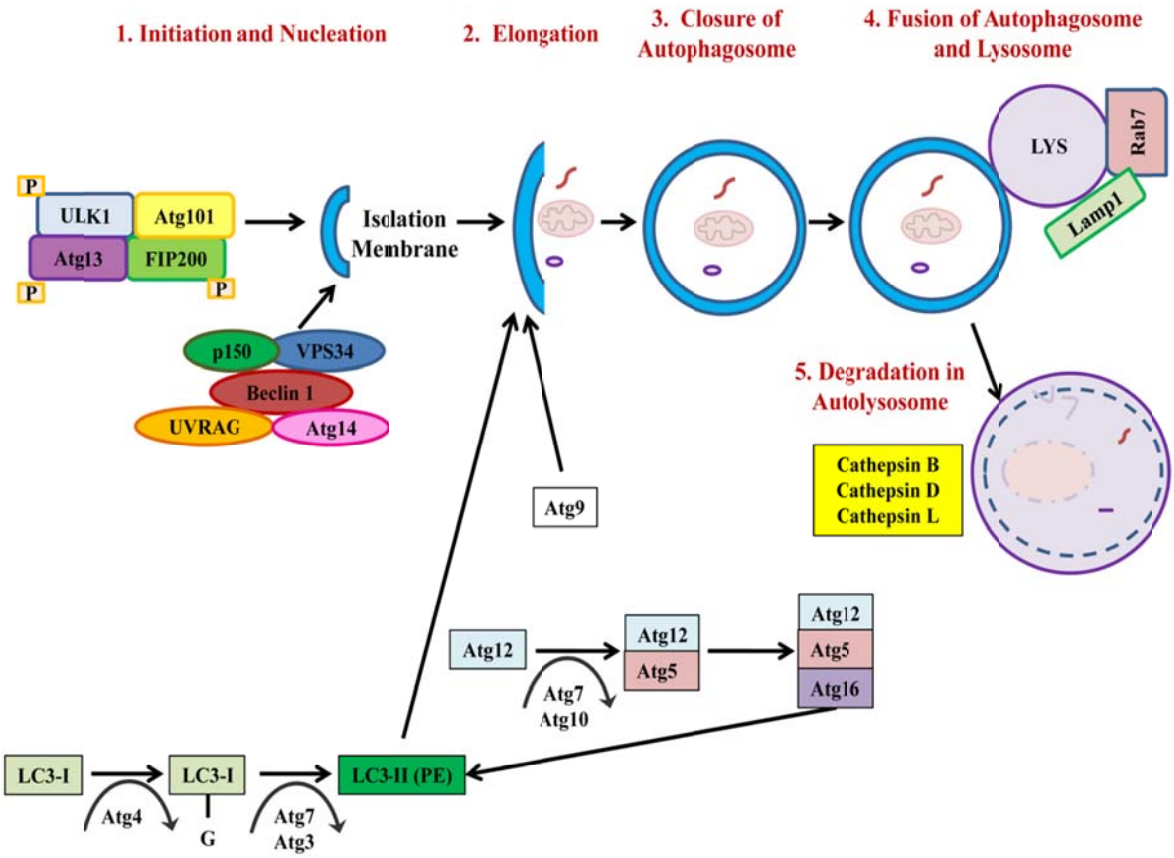

Figure 1.2.1B: Summary of Autophagy Machinery. 1) Autophagy is initiated by the ULK1 complex and nucleated to form the isolation membrane by the Beclin1/VPS34 complex. 2) Isolation membrane elongation occurs via two ubiquitin-like conjugation systems: The Atg5, Atg12, Atg16 complex and the LC3-II system. Atg9 also helps with elongation. 3) Mechanisms for autophagosome closure are still not understood. 4) Autophagosome-lysosome fusion requires the Rab7 GTPase and lysosomal membrane protein Lamp-1. 5) Degradation occurs via lysosomal hydrolases (Cathepsins B, D, and L).

1.2.2 History of Autophagy

The term “autophagy” is a combination of the Greek words “phagy” and “auto” meaning eat and self, respectively (Yang and Klionsky 2010). Christian de Duve discovered lysosomes in 1955, and he later described and coined the term “autophagy” after he observed single-membrane vesicles containing organelles or cytoplasm in rat hepatic cell lysosomes by electron microscopy (EM) in 1963 (De Duve 1963). He also discovered alongside others that autophagy could be induced by glucagon (Ashford and Porter 1962, De Duve and Wattiaux 1966, Deter, Baudhuin et al. 1967). The first inhibitors of autophagy were discovered in 1977. Pfeifer showed that insulin inhibits autophagy using EM analysis of rat liver cells (Pfeifer 1977), and Mortimore and Schworer showed that amino acids, specifically Leucine, Tyrosine, Phenylalanine, Glutamine, Proline, Histidine, Tryptophan, and Methionine, also inhibit autophagy in rat liver cells (Mortimore and Schworer 1977). Seglen and Gordon showed that Leucine was the strongest amino acid inhibitor of autophagy in 1980 (Seglen, Gordon et al. 1980). Seglen and Gordon also discovered the first pharmacological inhibitor of autophagy in 1982, which was 3-methyladenine (3-MA) (Seglen and Gordon 1982). However, its mechanism of action was not proven until 1997 when Meijer’s group showed that 3-MA inhibits autophagy by targeting Class III phosphatidylinositol 3-kinase (PI-3K), which is required for autophagosome formation (discussed in section 1.2.1). Meijer and colleagues also showed that Rapamycin (discussed in section 1.2.1) was an autophagy inducer in 1995 (Blommaart, Luiken et al. 1995).

Even though early studies leading to the discovery of autophagy were performed in the mammalian system, most of the studies that have contributed to our current knowledge of the autophagic process were performed in yeast. Dr. Ohsumi, who is known as the “Father of Autophagy”, discovered many important genes and their functions in the autophagy pathway using studies in yeast. Ohsumi’s group was the first to discover autophagy-related (*Atg*) genes

in yeast in 1993 using a genomic library screen (Tsukada and Ohsumi 1993), and they were also the first to describe functions of some of these genes in the autophagy pathway. In addition, Ohsumi's group was the first to show that the autophagy pathway in yeast is similar to that in mammalian cells (Tsukada and Ohsumi 1993, Mizushima, Sugita et al. 1998, Kabeya, Mizushima et al. 2000, Mizushima, Kuma et al. 2003). Dr. Beth Levine's group also contributed to this knowledge by identifying Beclin 1 as the mammalian homolog of yeast Atg6 in 1999 (Liang, Jackson et al. 1999). In 2000, LC3-II was shown to be on mammalian autophagosome membranes and was suggested to be used as a marker for detection of autophagy (Kabeya, Mizushima et al. 2000).

Even though Ohsumi discovered several autophagy genes in 1993, their function in the autophagy pathway was unknown for some time. In 1997, Ohsumi's group found that Atg1 is a kinase needed to initiate the autophagy pathway (Matsuura, Tsukada et al. 1997). Soon after, they along with Klionsky's group proved that a ubiquitin-like Atg12 conjugation system is also required for autophagosome formation (Mizushima, Noda et al. 1998, Mizushima, Sugita et al. 1998, Kim, Dalton et al. 1999). Ohsumi's group also later proved the importance of Atg8 lipidation for autophagosome formation (Ichimura, Kirisako et al. 2000) and showed that Atg8 is present on the autophagosome membrane (Kirisako, Baba et al. 1999). Finally in 2001, his group showed the importance of the Vps34 complex in the autophagy pathway (Kihara, Noda et al. 2001). Currently, there are over 30 known *Atg* genes.

1.2.3 Regulation of Mammalian Autophagy

Autophagy is activated by starvation, ER stress, hypoxia, proteasome inhibition, oxidative stress, protein aggregates, and pathogen infection (He and Klionsky 2009, Ding 2010, Ding, Manley et al. 2011). Mammalian target of rapamycin (mTOR) is an inhibitor of autophagy activation and is considered to be the master regulator of autophagy. mTOR is a

serine/threonine kinase important for maintaining cell growth, protein synthesis, and cellular homeostasis, and it is divided into two complexes including mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 activity is regulated by the phosphatidylionsital-3 kinase (PI3K) / protein-kinase B (AKT) signaling pathway, the AMP-activated protein kinase (AMPK) signaling pathway, and by the presence of amino acids in the lysosome. The PI3K / AKT pathway and lysosomal amino acids both result in activation of mTORC1 and inhibition of autophagy. The AMPK signaling pathway results in mTORC1 inhibition and autophagy activation. There is currently not a lot known about the mTORC2 complex, but it has been shown to be an activator of AKT, which is a positive regulator of mTORC1, as mentioned previously (Sarbassov, Guertin et al. 2005).

mTORC1 activation by the PI3K / AKT pathway requires insulin or growth factors, which bind the insulin receptor and lead to phosphorylation of AKT by PI3K. Activated AKT then phosphorylates and inactivates the tuberous sclerosis (TSC) tumor suppressor complex (TSC1/2), which is an mTORC1 inhibitor. TSC1/2 is a GTPase-activating protein (GAP) that inactivates the Ras homolog enriched in brain (Rheb) GTPase by converting it from the active GTP-bound form to the inactive GDP-bound form, and activated Rheb is needed for mTORC1 activation.TSC1/2 phosphorylation by AKT results in its inactivation and dissociation from the lysosome, and dissociation of TSC1/2 from the lysosome by AKT-mediated phosphorylation results in Rheb activation, leading to activation of mTORC1 (Inoki, Li et al. 2002, Potter, Pedraza et al. 2002, Inoki, Zhu et al. 2003, He and Klionsky 2009, Menon, Dibble et al. 2014).

mTORC1 activation by lysosomal amino acids is dependent on Rag GTPases (Rag A-D), which are part of the ragulator complex on the lysosome. When amino acids are present in the lysosome, Rag proteins are activated and form heterodimers comprised of an A/B (GTP-bound) subunit with a C/D (GDP-bound) subunit, which then bind to mTORC1 and recruit it to

the lysosome. Once on the lysosome, mTORC1 interacts with Rheb resulting in mTORC1 activation (Sancak, Bar-Peled et al. 2010).

AMPK, which is activated during low energy conditions by a high AMP/ATP ratio, inhibits mTORC1 signaling via phosphorylation of TSC2 in the TSC1/2 complex (Inoki, Zhu et al. 2003). In addition, AMPK can phosphorylate Raptor in the mTORC1 signaling pathway, which results in mTORC1 inactivation and autophagy activation (Gwinn, Shackelford et al. 2008). AMPK can also phosphorylate ULK1 on Ser317 and Ser 777, resulting in its activation and induction of autophagy (Kim, Kundu et al. 2011).

mTORC1 inhibits autophagy by attaching to the ULK1/2 complex (ULK1/2, ATG13, FIP200) in mammalian cells. Once attached, the mTORC1 kinase phosphorylates ULK1/2 on Ser757, which prevents ULK1-mediated phosphorylation of ATG13 and FIP200. ULK1/2 phosphorylation also disrupts the interaction between ULK1 and the positive autophagy regulator AMPK, resulting in autophagy inhibition. During starvation, mTORC1 dissociates from the ULK1/2 complex, allowing for autophagy activation (Jung, Jun et al. 2009, Kim, Kundu et al. 2011). mTORC1 also regulates autophagy by phosphorylating and inhibiting nuclear translocation of transcription factor EB (TFEB), which is a transcription factor important for inducing lysosome and autophagy-specific genes. During starvation, the dephosphorylated form of TFEB is able to translocate to the nucleus and induce transcription of these genes (Settembre, Zoncu et al. 2012). A summary of mTOR-dependent autophagy regulation is shown in **Figure 1.2.3**. Autophagy can also be regulated by mTOR-independent mechanisms. For example, autophagy is regulated by the transcription factor FoxO3 (Forkhead box transcription factor class O3) (Mammucari, Milan et al. 2007, Ni, Du et al. 2013).

Figure 1.2.3

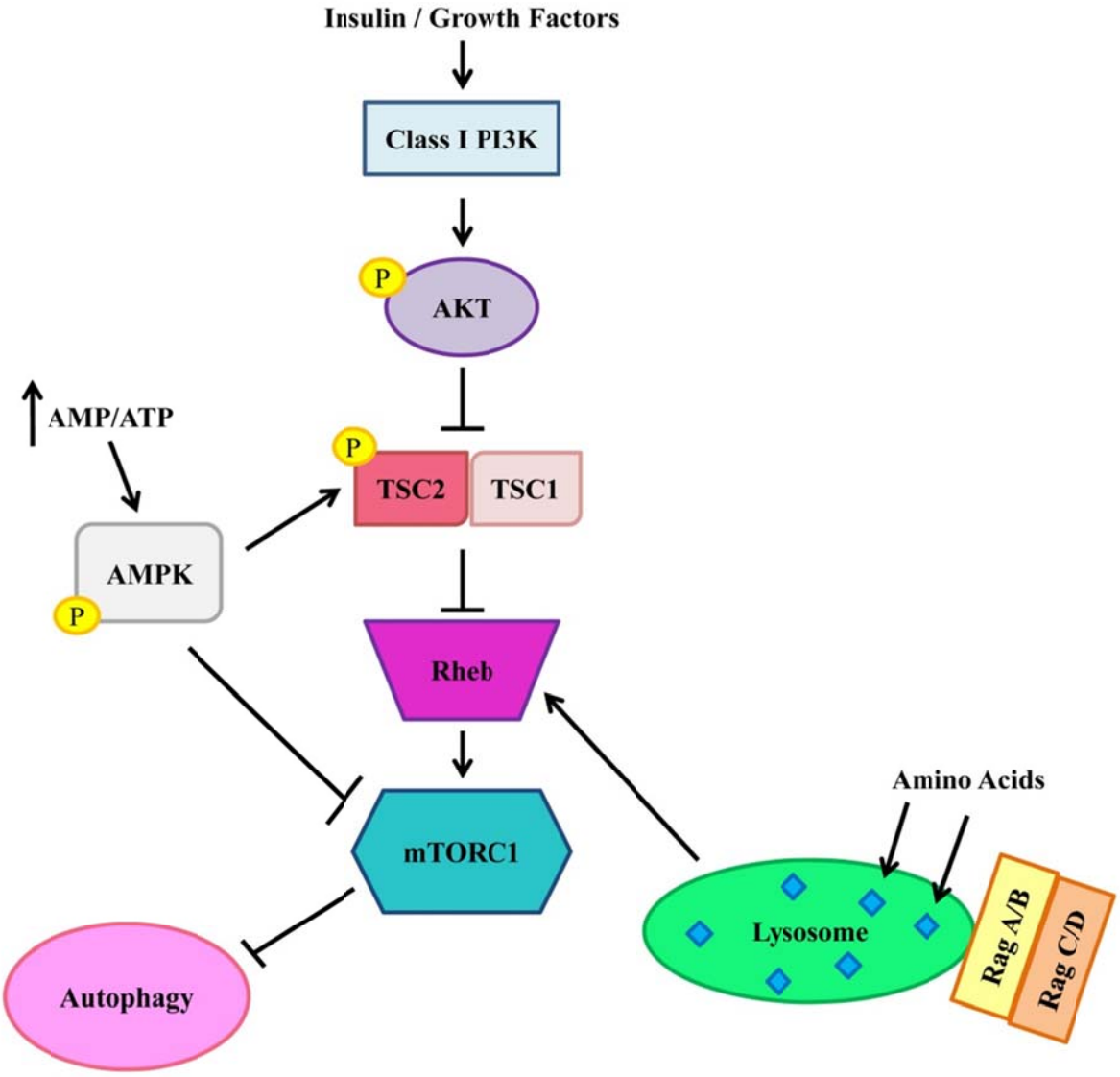

Figure 1.2.3: Regulation of mTORC1 by AKT, AMPK and lysosomal amino acids. After binding of growth factors, AKT activates mTORC1 by inhibiting the TSC1/TSC2 complex, which results in Rheb activation. Lysosomal amino acids activate mTORC1 by activating Rheb. During low energy conditions, AMPK inhibits mTORC1 activation by phosphorylating TSC2, resulting in Rheb inhibition. mTOR activation results in autophagy inhibition.

1.2.4 Pharmacological Modulation of Autophagy

Pharmacological modulators of autophagy are of great interest due to the role of autophagy in many pathological conditions such as cancer, drug-induced liver injury, neurodegenerative diseases, pathogenic infections, cardiovascular disease, liver disease, and aging (Ding 2010, Kroemer 2015). In addition, one of the key methods for studying autophagy involves its pharmacological modulation. Common drugs used for autophagy manipulation include drugs that target mTORC1 and drugs that are mTORC1-independent and target AMPK, phosphatidylinositol, and lysosome function. Several drug screens for determining new autophagy modulators have been completed or are currently ongoing, but the effects on autophagy by drugs found in these screens still need to be confirmed (Rubinsztein, Codogno et al. 2012).

The role of mTOR in autophagy regulation was discussed in section 1.2.3. mTOR-dependent modulators for autophagy activation include rapamycin (binds FK-506 and inhibits mTORC1), torin 1 (inhibits MTORC1 and mTORC2 kinase activity), and metformin (inhibits mTORC1 and activates ULK1 via AMPK activation), among others (Kim and Guan 2015, Vakifahmetoglu-Norberg, Xia et al. 2015). mTOR-independent inhibitors of autophagy include the PI3K inhibitors 3-Methyladenine (3-MA) and wortmannin, the calcium channel antagonist thapsigargin, and inhibitors of lysosomal degradation such as chloroquine and Bafilomycin A, which cause a rise in lysosomal pH. E64d, pepstatin A, and leupeptin also inhibit autophagosome degradation through inhibition of lysosome hydrolases, proteases, and peptidases (Vakifahmetoglu-Norberg, Xia et al. 2015).

1.2.5 Mechanisms for Detection of Autophagy Induction

Common methods for determining autophagy activation include analysis of autophagosome formation by electron microscopy (EM) and quantification of LC3-II and Sequestosome 1 /p62 protein levels by Western blot. Fluorescence microscopy for LC3 puncta, which are representative of autophagosome formation, is another commonly used method. In this dissertation, autophagosomes and lysosomes along with their contents were identified and quantified by EM. Initial autophagosomes (AVi) can be identified by their double membrane, and they contain intact organelles or cytosol. Late-stage autolysosomes (AVd) can be identified by a single membrane, and they contain organelles that are partially degraded (Klionsky, Abdalla et al. 2012) (**Figure 4.4.5 G and J**). Quantification of LC3-II protein levels by Western blot was also used in this dissertation for determining autophagy activation. Both the lipidated (LC3-II, 16 kDa) and delipidated (LC3-I, 18 kDa) forms of LC3 can be detected by western blot, but the lipidated LC3-II form is the only form that is present on mature autophagosomes, which is why it is commonly used as a marker of autophagy activation (Klionsky, Abdalla et al. 2012). In addition, determination of p62 protein levels by western blot was used for determining autophagy activation because p62 is degraded in the lysosome during autophagy (Manley, Williams et al. 2013). The role of p62 in autophagy is further discussed in section 1.3.2.

The best approach for monitoring autophagy activation involves using these methods in combination with an autophagy flux assay. It is now widely accepted that proper analysis of autophagy activation requires an autophagy flux assay, which takes all parts of the autophagy pathway from autophagosome formation to degradation of cargo in the lysosome into account. The autophagy pathway is dynamic and involves many steps, which were previously discussed in section 1.2.1. Many of these steps can be activated or inhibited, causing results interpreted without an autophagy flux assay to be questionable. For example, an increase in LC3-II protein levels can be due to either increased autophagosome formation or decreased autophagosome

degradation in the lysosome. Therefore, assays used for determining autophagy activation, such as EM and quantification of LC3-II levels, should always be performed along with an autophagy flux assay to accurately determine autophagy activation or inhibition. In this dissertation, chloroquine (CQ) was used for all autophagy flux assays. CQ inhibits degradation of cargo in the lysosome by raising lysosome pH. When treatment with an autophagy inducer is combined with CQ, LC3-II levels, or the number of autophagosomes, will be increased compared to treatment with the inducer or CQ alone (Klionsky, Abdalla et al. 2012).

1.2.6 Protective Roles of Autophagy in the Liver

Autophagy plays several important roles in the liver in order to maintain homeostasis and cell survival. One important role of autophagy in the liver is degradation of damaged organelles and aggregated proteins. Hepatocellular injury and death would occur if cellular components such as lipids, aggregated proteins, and damaged or excess organelles were allowed to accumulate over time in the absence of autophagy (Czaja, Ding et al. 2013) .

Autophagy in the liver also allows for energy production during starvation. During starvation, autophagy in the liver provides amino acids and substrates needed for energy production and hepatocellular survival. At basal levels, autophagy degrades approximately 1.5% of hepatic protein in one hour. However, in starvation conditions, this level of hepatic protein degradation increases to 4.5% (Schworer, Shiffer et al. 1981).

Autophagy also helps regulate fat accumulation in the liver by degrading lipid droplets, which provides free fatty acids that can then be used in the β-oxidation pathway for energy production. The process of degradation of fat in the liver via autophagy is a selective from of autophagy termed lipophagy (Singh, Kaushik et al. 2009, Liu and Czaja 2013).

Autophagy also has several important roles in protection from liver diseases. The role of autophagy in protection against alcoholic liver disease (ALD) and drug-induced liver injury by acetaminophen are extensively discussed in sections 3 and 4, respectively.

1.3 Selective Autophagy with a Focus on Mitophagy for Degradation of Damaged Mitochondria

Selective autophagy occurs in nutrient rich or poor conditions and functions to isolate and degrade damaged proteins and organelles (Reggiori, Komatsu et al. 2012). Different types of selective autophagy are named according to the cargo they degrade. Reticulophagy or ERphagy is for degradation of endoplasmic reticulum (Hamasaki, Noda et al. 2005), ribophagy is for degradation of ribosomes (Kraft, Deplazes et al. 2008), xenophagy is for degradation of pathogens (Levine 2005), pexophagy is for degradation of peroxisomes (Dunn, Cregg et al. 2005), lipophagy is for degradation of lipid droplets (Singh, Kaushik et al. 2009), aggrephagy is for degradation of protein aggregates (Yamamoto and Simonsen 2011), and mitophagy, the subject of this dissertation, is the selective degradation of damaged mitochondria by autophagy (Lemasters 2005, Narendra, Tanaka et al. 2008). John Lemasters gave “mitophagy” its name in 2005 (Lemasters 2005), but mitochondria were observed in lysosomes by Ashford and Porter in 1962 (Ashford and Porter 1962). Mechanisms of mitophagy induction have been well studied in yeast and in mammalian cells. However, most have not been studied in the liver. There are several mechanisms for mitophagy induction in mammalian systems including both Parkin-dependent (discussed in section 1.3.2) and Parkin-independent pathways (discussed in section 5.1.1). In addition, mitochondrial dynamics (fission and fusion) are important for proper mitophagy induction.

1.3.1 Mitochondrial Fission and Fusion are Important for Mitophagy

Mitochondria are dynamic organelles that continuously undergo fission and fusion, and the length of mitochondria is determined by their fission and fusion rates. Mitochondria can be either a large network of fused organelles or they can be divided into many smaller fragments depending on the needs of the cell. These fission and fusion events are necessary for cell

survival because they allow the cell to adapt to changing conditions needed for cell growth, division, and distribution of mitochondria during differentiation (van der Bliek, Shen et al. 2013). Mitochondrial fission and fusion also allow the cell to adapt to injury because damaged mitochondria can segregate from healthy mitochondria, leading to degradation of damaged mitochondria by mitophagy and fusion of healthy mitochondria (Twig, Elorza et al. 2008).

Mitochondrial fusion in mammals is mediated by the pro-fusion genes mitofusin 1 and mitofusin 2 (MFN1/2) and optic atrophy 1 (OPA1). MFN1/2 are GTPases that are responsible for fusion of outer mitochondrial membranes, and OPA1 is a dynamin-related GTPase responsible for fusion of inner mitochondrial membranes. Outer and inner mitochondrial membrane fusions mostly occur simultaneously (Westermann 2010, van der Bliek, Shen et al. 2013). Mitochondrial fission in mammals is mediated by dynamin-related protein 1 (Drp1), which is a cytosolic protein that can be recruited to the surface of mitochondria where it interacts with mitochondrial fission 1 (Fis1) to initiate mitochondrial fission (Westermann 2010). It has been suggested that mitochondria fission factor (Mff), which is an outer mitochondrial membrane protein that interacts with Drp1, is an essential factor for Drp1-mediated mitochondrial fission (Gandre-Babbe and van der Bliek 2008, Otera, Wang et al. 2010). In addition, mitochondria and endoplasmic reticulum are often tightly associated and in physical contact. It was reported that mitochondrial fission proteins, such as Drp1, are localized at the endoplasmic reticulum-mitochondria contact site and that the endoplasmic reticulum may play a role in the process of mitochondrial fission (Friedman, Lackner et al. 2011).

It has been suggested that fragmented mitochondria are more easily engulfed by autophagosomes during mitophagy. In addition, elongated mitochondria are spared from autophagosome sequestration (Ding and Yin 2012). After photo-labeling, mitochondria underwent continuous cycles of fission and fusion, and fission events resulted in two sets of daughter mitochondria with either increased or decreased membrane potential (Twig, Elorza et

al. 2008). Daughter mitochondria with higher membrane potential proceeded to fusion while depolarized daughter mitochondria were degraded by mitophagy (Twig, Elorza et al. 2008). Therefore, mitochondrial fission is a necessary step for mitophagy induction. However, mitochondrial fission alone is not enough to induce mitophagy. Mitochondria must be both damaged/depolarized and fragmented and may also possibly need to recruit other autophagy receptor proteins for mitophagy induction to occur (Ding and Yin 2012). In addition, excessive fusion of mitochondria has been shown to inhibit the mitophagy process (Twig and Shirihai 2011), further suggesting an importance of mitochondrial fragmentation as a prerequisite to mitophagy induction.

1.3.2 Parkin-dependent Mitophagy

Parkin is encoded by the *Park2* gene and is a 465 amino acid protein (Kitada, Asakawa et al. 1998). Parkin is well known for its protective role in the brain because loss of Parkin plays a role in development of Autosomal Recessive Parkinson's disease, and the *Park2* gene was discovered in 1997 by Mizuno's group as an unidentified gene responsible for this disease (Matsumine, Saito et al. 1997). The gene was cloned in 1998 by Shimizu's group (Kitada, Asakawa et al. 1998). Approximately 10% of early onset Parkinson's disease cases are caused by mutations in the *Park2* gene (Houlden and Singleton 2012), and gene mutations can occur throughout the *Park2* gene and cause loss of Parkin function (Wauer and Komander 2013, Seirafi, Kozlov et al. 2015). In addition to genetic mutations, Parkin can also be inactivated post-translationally via oxidation, nitrosylation, or phosphorylation (Walden and Martinez-Torres 2012). Even though the majority of research regarding Parkin has been related to neurodegenerative diseases such as Parkinson's disease, we found that Parkin is also highly expressed in the liver in mice (Ding and Yin 2012).

Parkin is an E3 ubiquitin ligase (Shimura, Hattori et al. 2000). The mechanism for ubiquitination by E3 ligases was discussed in section 1.1. Parkin contains several domains including a UBL (ubiquitin-like) domain, which is recognized by the proteasome, and three RING (really interesting new gene) domains (RING0, RING1, and RING2) in addition to an IBR (in between RING) domain (**Figure 1.3.2A**). The RING1 domain contains an E2 ligase binding site, the RING2 domain contains a catalytic cysteine residue (Cys431) necessary for transfer of ubiquitin from Parkin to substrate, and the RING0 domain is a zinc-finger domain (Hristova, Beasley et al. 2009, Riley, Lougheed et al. 2013). Parkin is a RING-HECT (RING and HECT domains discussed in section 1.1) hybrid E3 ligase. Parkin does not have a HECT domain, but it transfers ubiquitin to substrate using a combination of mechanisms from RING and HECT E3 ligases. To ubiquitinate a substrate protein, Parkin first transfers ubiquitin from an E2 ligase to its RING1 domain and then moves it to its RING2 domain. The ubiquitin is transferred within the RING2 domain to a catalytic Cys431 residue before being further transferred to a lysine amino group on the substrate protein (Wenzel, Lissounov et al. 2011, Riley, Lougheed et al. 2013, Trempe, Sauve et al. 2013, Zheng and Hunter 2013).

Parkin is well known to induce mitophagy in *in vitro* systems after treatment with the mitochondrial uncoupler Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) via ubiquitination of outer mitochondrial membrane proteins. Parkin was originally found to be involved in the mammalian mitophagy pathway in 2008 by Richard Youle's group (Narendra, Tanaka et al. 2008). Parkin translocated from the cytosol to depolarized mitochondria in Parkin-expressing HEK293 cells and in HeLa cells with YFP-Parkin overexpression after CCCP treatment. In addition, Parkin preferred depolarized mitochondria to healthy mitochondria because Parkin only translocated to depolarized mitochondria after CCCP treatment, which was shown using MitoTracker Red staining, a dye used to analyze mitochondrial polarization. HeLa cells with lower MitoTracker Red intensity, indicative of depolarization, had a greater accumulation of

YFP-Parkin expression after CCCP treatment. Furthermore, mitochondrial mass was decreased in Parkin-overexpressing HeLa cells after CCCP treatment but unchanged in non-Parkin expressing HeLa cells, suggesting that Parkin recruitment to depolarized mitochondria induced their degradation. Finally, Parkin co-localized with the autophagosome marker LC3 after CCCP treatment, indicating that Parkin-induced degradation of mitochondria was via mitophagy. Parkin also selectively translocated to damaged mitochondria while avoiding healthy mitochondria, which was analyzed by fluorescence loss in photobleaching (FLIP) in YFP-Parkin expressing HeLa cells (Narendra, Tanaka et al. 2008).

Parkin-dependent mitophagy requires both Parkin and PINK1 (phosphatase and tensin homolog (PTEN)-induced kinase 1). PINK1 is upstream of Parkin in this pathway because overexpression of Parkin in PINK1-deficient *Drosophila* partially rescued the PINK1 mutant phenotype while overexpression of PINK1 failed to do so in Parkin-deficient *Drosophila* (Clark, Dodson et al. 2006, Park, Lee et al. 2006, Yang, Gehrke et al. 2006). In addition, Parkin directly interacts with PINK1 (Um, Stichel-Gunkel et al. 2009), and overexpression of PINK1 alone can initiate translocation of Parkin to mitochondria without mitochondrial damage (Kawajiri, Saiki et al. 2010). The specificity for PINK1's role in recruiting Parkin was further shown by ectopic expression of PINK1 on peroxisomes, which resulted in recruitment of Parkin and degradation of the PINK1-expressing peroxisomes by autophagy (Lazarou, Jin et al. 2012).

PINK1 promotes Parkin-mediated mitophagy by recruiting Parkin to damaged mitochondria. PINK1 also activates Parkin's E3 ligase activity to induce ubiquitination of mitochondrial outer membrane proteins and subsequent degradation of mitochondria in autophagosomes. PINK1 is normally cleaved and degraded in healthy mitochondria. However, PINK1 becomes stabilized on the outer mitochondrial membrane when mitochondria are depolarized, which signals Parkin recruitment to damaged mitochondria. To induce PINK1 cleavage in healthy mitochondria, the transporter outer membrane (TOM) complex on the outer

mitochondrial membrane imports PINK1 into the inner mitochondrial membrane through the transporter inner membrane (TIM) complex where it is degraded by PARL (presenilin-associated rhomboid-like protein) (Jin, Lazarou et al. 2010). Cleavage by PARL removes the N-terminus of PINK1, and the truncated PINK1 protein is released into the cytosol where it is degraded by the UPS (Yamano and Youle 2013). However, when mitochondria are depolarized, import of PINK1 into the inner mitochondrial membrane is blocked and PINK1 is no longer cleaved and degraded and instead becomes stabilized on the outer mitochondrial membrane (Jin, Lazarou et al. 2010). Accumulation of misfolded proteins in the mitochondria can also stabilize PINK1 on the outer mitochondrial membrane without mitochondrial depolarization. However, the mechanism for PINK1 stabilization in this model is unknown (Jin and Youle 2013). In addition to its stabilization on the outer mitochondrial membrane, PINK1 must also phosphorylate itself on Serine228 and Serine204 to recruit Parkin to mitochondria (Okatsu, Oka et al. 2012).

In addition to PINK1, several other genes were found to be important for regulation of Parkin mitochondrial translocation using a genome-wide small interfering RNA (siRNA) screening. Translocase of outer mitochondrial membrane 7 (TOMM7) stabilizes PINK1 on the outer mitochondrial membrane whereas seven in absentia homolog 3 (SIAH3), a mitochondrial resident protein, de-stabilizes PINK1 on mitochondria. Furthermore, two other proteins, HSPA1L (an HSP70 family protein) and Bcl2-associated athanogene 4 (BAG4, a nucleotide exchange factor for HSP70), positively and negatively regulate Parkin mitochondrial translocation, respectively (Hasson, Kane et al. 2013).

Parkin, unless activated, remains in an auto-inhibited state by several mechanisms, preventing its transfer of ubiquitin to substrate. The RING0 domain blocks the RING2 domain, preventing ubiquitin transfer. The UBL domain of Parkin also blocks the E2 binding site on the RING1 domain, preventing acceptance of ubiquitin from an E2 ligase. In addition, there is

distance between the E2 binding site and the E2 catalytic Cys431 residue within the RING2 domain that requires a conformational change to allow for ubiquitin transfer from E2 to E2-Cys431 (Riley, Lougheed et al. 2013, Trempe, Sauve et al. 2013, Wauer and Komander 2013). PINK1-mediated phosphorylation of Parkin activates Parkin's E3 ligase activity. PINK1 phosphorylates Ser65 in Parkin's UBL domain (Kondapalli, Kazlauskaite et al. 2012, Shiba-Fukushima, Imai et al. 2012, Iguchi, Kujuro et al. 2013), which promotes a conformational change in Parkin and activation of its E3 ligase activity allowing it to transfer ubiquitin from its E2 binding site to its E2-Cys431 residue and finally to its targeted outer mitochondrial membrane protein (Iguchi, Kujuro et al. 2013). It was also recently shown that PINK1 phosphorylates ubiquitin at Ser65. Ser65-phosphorylated ubiquitin binds to Ser65-phosphorylated Parkin with 21-fold greater affinity compared to non-phosphorylated Parkin, resulting in greater levels of Parkin activity and mitochondrial protein ubiquitination (Kane, Lazarou et al. 2014, Koyano, Okatsu et al. 2014). PINK1-induced phosphorylation of both Parkin and Ubiquitin at Ser65 acts as a feed-forward loop for ubiquitination of substrate proteins on the outer mitochondrial membrane. Once Parkin is recruited to the mitochondria and activated by Ser65 phosphorylation, PINK1 phosphorylates ubiquitin (Ser65) attached to mitochondrial proteins by Parkin, which tethers Parkin to the mitochondria allowing for further ubiquitination of outer mitochondrial membrane proteins by Parkin to initiate mitophagy (Ordureau, Sarraf et al. 2014, Shiba-Fukushima, Arano et al. 2014).

Parkin ubiquitinates several mitochondrial outer membrane proteins through lysine48 and lysine63 ubiquitin linkages (Chan, Salazar et al. 2011) with the help of four known E2 ligases: UBE2L3, UBE2D2, UBE2D3, and UBE2N (Geisler, Vollmer et al. 2014). Harper's group found 36 outer mitochondrial membrane proteins that are Parkin substrates (Sarraf, Raman et al. 2013). The most studied of these substrates for mitophagy initiation include the mitochondrial fusion proteins MFN1/2, the mitochondrial trafficking protein Miro1, Translocase of outer

mitochondrial membrane 20 (TOM20), and Voltage-dependent anion channel (VDAC). Ubiquitination of MFN1/2 initiates their subsequent degradation by the proteasome resulting in mitochondrial fission and fragmentation (Gegg, Cooper et al. 2010, Geisler, Holmstrom et al. 2010, Poole, Thomas et al. 2010, Chan, Salazar et al. 2011). Mitochondria are under continuous states of fission and fusion, and mitochondrial fission is important for mitophagy induction in liver hepatocytes among other cell types as discussed in section 1.3.1 (Twig, Elorza et al. 2008, Kim and Lemasters 2011, Ding and Yin 2012). MFN2 may have a role in mediating Parkin recruitment to damaged mitochondria in addition to PINK1 (Chen and Dorn 2013). Parkin-induced ubiquitination of Miro initiates mitochondrial arrest, which can segregate damaged mitochondria from healthy mitochondria prior to mitophagy (Wang, Winter et al. 2011). Parkin-induced ubiquitination of VDAC leads to recruitment of the autophagy adaptor protein p62 to the mitochondria (Geisler, Holmstrom et al. 2010). p62 contains both a ubiquitin-associated domain (UBA) and also an LC3-interacting region (LIR), which allows it to bind to ubiquitinated proteins and organelles and transport them to autophagosomes for degradation by binding to LC3 on the autophagosome membrane (Manley, Williams et al. 2013). The role of p62 in mitophagy is currently controversial because some have shown it is required for mitophagy (Ding, Ni et al. 2010, Geisler, Holmstrom et al. 2010), while others have shown that it is not (Narendra, Kane et al. 2010, Okatsu, Saisho et al. 2010). However, BNIP3L/Nix is also a Parkin substrate, and Parkin-mediated ubiquitination of Nix recruits the autophagy adaptor protein Neighbor of BRCA Gene 1 (NBR1) to damaged mitochondria to help shuttle them to the autophagosome for degradation (Gao, Chen et al. 2015). Therefore, NBR1 and/or p62 may act in the mitophagy pathway to degrade damaged mitochondria. In addition, K48 ubiquitination of specific outer mitochondrial membrane proteins by Parkin induces their degradation via the proteasome, which causes mitochondrial fragmentation and may improve mitophagy efficiency by segregating mitochondria into smaller pieces so they can be more easily engulfed by

autophagosomes (Chan, Salazar et al. 2011, Yoshii, Kishi et al. 2011). However, the exact mechanism for how autophagosomes are recruited to damaged mitochondria after Parkin-induced ubiquitination of outer mitochondrial membrane proteins is unknown. Parkin-mediated induction of mitophagy is shown in **Figure 1.3.2B.**

Other proteins involved in activation of the Parkin-mediated mitophagy pathway are Nix and Smurf1. In addition to its role in recruiting NBR1, Nix also promotes the recruitment of Parkin to depolarized mitochondria after CCCP treatment in MEF cells. CCCP treatment caused mitochondrial depolarization, ROS accumulation, and translocation of Parkin to mitochondria in Parkin-expressing MEF cells while NIX-deficient MEF cells failed to induce mitochondrial depolarization and Parkin recruitment, suggesting that NIX may also be required for Parkin-dependent mitophagy induction (Ding, Ni et al. 2010). SMURF1 is an E3 ubiquitin ligase similar to Parkin that was found to have a role in CCCP-induced mitophagy mediated by Parkin. Unlike Parkin, the ubiquitin ligase function of SMURF1 does not play an important role in mitophagy induction. Instead, the C2 domain of SMURF1 is required for engulfment of damaged mitochondria by autophagosomes. In addition, SMURF1-deficient mice have accumulated mitochondria that are damaged in their heart, brain, and liver (Orvedahl, Sumpter et al. 2011).

Parkin-induced mitophagy is negatively regulated by ubiquitin-specific peptidases 30 (USP30) and USP15, which are deubiquitinases localized to mitochondria that inhibit Parkin-mediated mitophagy by removing ubiquitin from damaged mitochondria previously attached by Parkin. Knockdown of USP30 rescued defective mitophagy caused by pathogenic mutations in Parkin and improved mitochondrial integrity in Parkin- or PINK1-deficient flies, protecting them against paraquat toxicity (Bingol, Tea et al. 2014). Knockdown of USP15 in *Park2* mutated human fibroblasts rescued mitophagy and also protected from mitochondrial defects in *Drosophila* (Cornelissen, Haddad et al. 2014). Parkin has also been shown to ubiquitinate itself to initiate its own degradation (Zhang, Gao et al. 2000).

Figure 1.3.2A

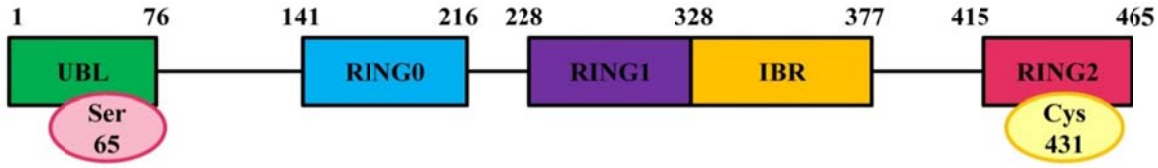

Figure 1.3.2A: Structure of Parkin. Parkin contains a UBL domain at its N-terminus, which is recognized by the proteasome. Ser65 in the UBL domain is also phosphorylated by PINK1 to activate Parkin's E3 ligase activity, which is necessary for Parkin to transfer ubiquitin to a substrate protein. The RING0 domain is a zinc-finger domain. The RING1 domain is the binding site for an E2 ligase. The RING2 domain contains the catalytic Cys431 residue needed for Parkin to transfer ubiquitin to substrate.

Figure 1.3.2B

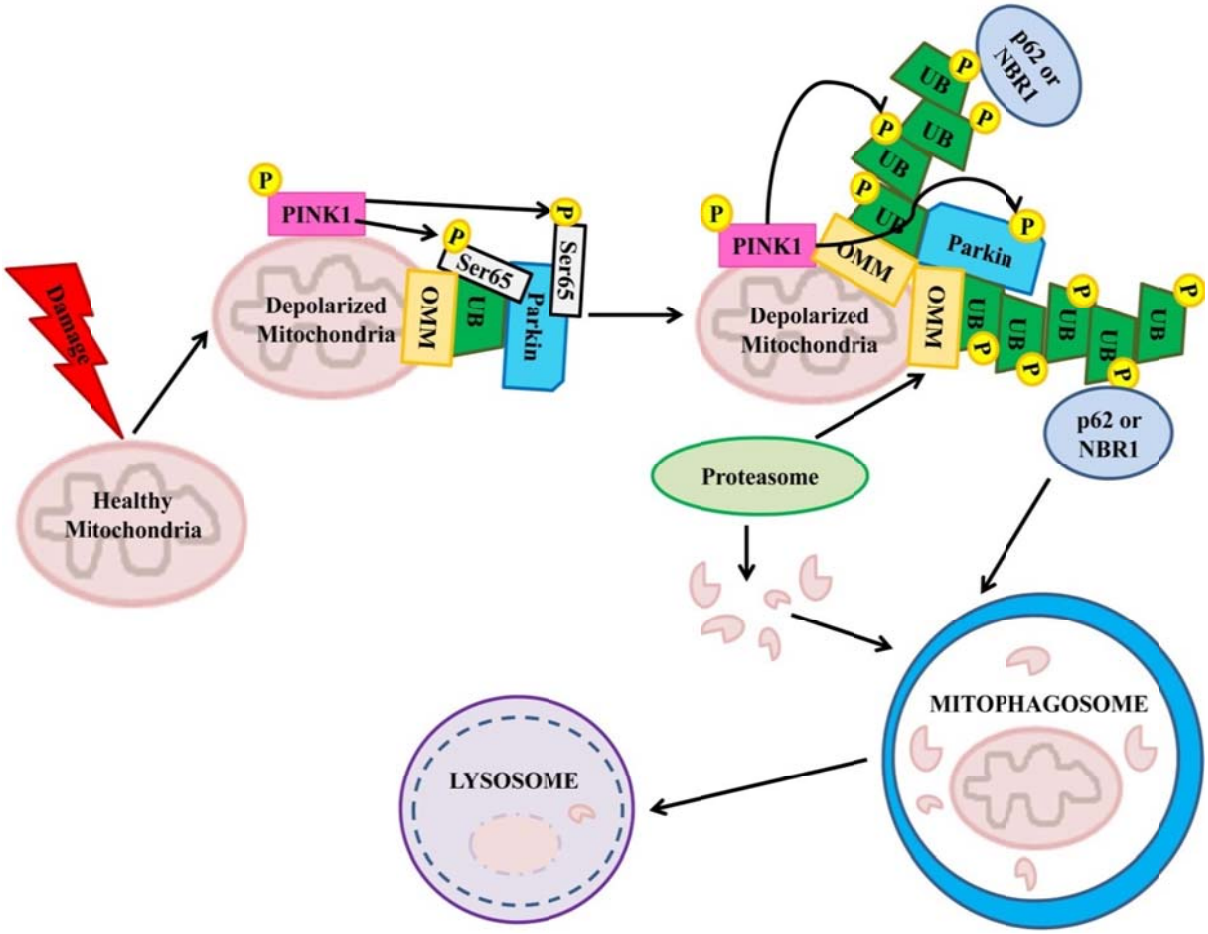

Figure 1.3.2B: Mechanism for Parkin-induced Mitophagy. When mitochondria are damaged/depolarized, PINK1 is stabilized on the outer mitochondrial membrane where it auto-phosphorylates itself on Ser228 and Ser402, signaling Parkin recruitment to the mitochondria. Once on the mitochondria, PINK1 phosphorylates Parkin at Ser65, causing a conformational change in Parkin so it can attach its ubiquitin (UB) onto an outer mitochondrial membrane substrate. PINK1 then phosphorylates this UB on Ser65, increasing binding affinity of Parkin for UB and tethering it to the mitochondria. This becomes a feed-forward loop allowing for Parkin to polyubiquitinate outer mitochondrial membrane proteins. Autophagy adaptor proteins, such as p62 and/or NBR1, can bind to ubiquitinated proteins on the mitochondria, resulting in autophagosome recruitment to damaged mitochondria and their eventual degradation in the lysosome. The proteasome is also thought to help with mitochondrial degradation in autophagosomes by degrading lysine48-ubiquitinated outer mitochondrial membrane proteins, leading to mitochondrial fragmentation and easier engulfment of mitochondria by the autophagosome.

1.3.3 Methods for Mitophagy Detection

EM and Western blot analysis were used for mitophagy analysis in this dissertation. EM is an easy method for detection of mitophagy induction via visualization of mitochondria in autophagosomes and autolysosomes. Mitochondria present in autophagosomes are identified by their density and cristae, while mitochondria in autolysosomes are identified by their density along with the occasional presence of their membrane structure. However, this method is not very quantitative because it only allows visualization of a few cells.

Mitophagy can also be assessed by Western blot analysis for mitochondrial protein degradation along with changes in the autophagy markers LC3-II and p62 (previously discussed in section 1.2.5). For mitochondrial protein degradation, it is important to look at several mitochondrial proteins from the outer membrane, inner membrane, and matrix because they have been shown to be degraded by different systems at different rates. Mitochondrial membrane proteins are degraded via the proteasomal system while the mitochondrial matrix proteins are removed by autophagy (Chan, Salazar et al. 2011, Yoshii, Kishi et al. 2011). However, not all matrix proteins are removed by autophagy. It is still unclear how autophagy selectively removes some matrix proteins but not others, but Abeliovich *et al.* showed that different mitochondrial matrix proteins are degraded at distinct different rates during yeast mitophagy. They found that mitochondria underwent an active matrix protein segregation process, which is regulated by the yeast fission molecule Dynamin-1. In addition, the rates of mitophagic degradation of matrix proteins correlate with the degree of physical segregation of specific matrix proteins (Abeliovich, Zarei et al. 2013) These findings suggest that mitochondrial dynamics are important for mitochondrial matrix remodeling/segregation, which may help to explain why mitochondrial proteins are degraded at different rates by mitophagy. In addition to mitochondrial protein degradation, mitochondrial and cytosolic protein extracts can be used to look for increases in p62 and LC3-II protein expression in the mitochondrial fraction, which hints

at induction of mitophagy. When using p62 and LC3-II as mitophagy markers, it is best to use an autophagic flux assay (previously discussed in section 1.2.5). When monitoring occurrence of mitophagy, it is important to use a combination of techniques to overcome pitfalls of currently available assays. More reliable assays are needed for quantification and detection of mitophagy (Ding and Yin 2012, Zhu, Chen et al. 2014).

1.4 Mitophagy Protects against Liver Injury

Mitochondria are considered the “power house” of the cell due to being the major site of ATP production. Mitochondria also have other functions including heme synthesis, β-oxidation of fatty acids, and maintenance of calcium homeostasis (Duchen 2004). It is well known that mitochondria act as central executioners of cell death including apoptotic and necrotic cell death. As a result, mitochondrial dysfunction is a key event in progression of liver injury and disease due to accumulation and release of damaging reactive oxygen species (ROS), decreased oxidative phosphorylation and impaired ATP synthesis, and release of pro-apoptotic proteins or opening of their mitochondrial permeability transition pore for initiation of cell death by apoptosis or necrosis. Mitochondrial damage is reflected by decreased respiratory parameters, decreased enzyme activity, accumulation of mitochondrial DNA mutations, and increased oxidative stress (Hill, Benavides et al. 2012). Mitochondrial dysfunction plays a role in progression of many different types of liver disease such as alcoholic liver disease, non-alcoholic liver disease, Hepatitis C, cholestasis (Grattagliano, Russmann et al. 2011) and drug-induced liver injury (Han, Dara et al. 2013).

Mitochondria can maintain homeostasis through many different mechanisms. They have their own proteolytic system, allowing them to degrade misfolded proteins that could potentially disrupt mitochondrial function (Baker and Haynes 2011, Matsushima and Kaguni 2012). In addition, outer mitochondrial membrane proteins that are damaged can be degraded by the proteasome (Karbowski and Youle 2011). Mitochondria can also repair damaged components via constant fission and fusion (further discussed in section 1.3.1), which allows for segregation of damaged mitochondria and exchange of material between healthy mitochondria (Twig, Elorza et al. 2008, van der Bliek, Shen et al. 2013). Furthermore, mitochondria-derived vesicles can bud off of mitochondria and fuse with lysosomes to degrade their cargo (further discussed in section 1.5.1) (Soubannier, Rippstein et al. 2012) or damaged mitochondria can form

mitochondrial spheroids and acquire lysosomal markers to possibly serve as an alternative pathway for removal of damaged mitochondria (further discussed in section 1.5.2) (Ding, Guo et al. 2012, Ding, Li et al. 2012, Yin and Ding 2013). Finally, mitophagy (discussed in section 1.3) initiates engulfment of damaged mitochondria by autophagosomes to trigger their degradation in the lysosome (Youle and Narendra 2011, Ding and Yin 2012, Lemasters 2014), which is an important mechanism for protection against liver injury. Mitophagy is activated by many different cellular stress conditions including loss of mitochondrial membrane potential, changes in mitochondrial bioenergetics, accumulation of cellular ROS, mitochondrial DNA damage, or accumulation of protein aggregates in the mitochondria (Liu, Sakakibara et al. 2014). Removing damaged mitochondria by mitophagy prevents the spread of oxidative stress, respiratory chain damage and mitochondrial DNA mutation, which helps to prevent cell death, maintain mitochondrial bioenergetics, and uphold fatty acid oxidation by preserving a healthy population of mitochondria.

1.4.1 Mitophagy Prevents Apoptosis and Necrosis

Hepatocyte cell death via either apoptosis or necrosis worsens liver disease and injury progression. Cell death by apoptosis in the liver plays major roles in progression of cholestasis, alcoholic liver disease, Hepatitis C, and fibrosis (Luedde, Kaplowitz et al. 2014). Apoptosis is a method of programmed-cell death that is characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and cellular fragmentation into membrane-enclosed and organelle-containing apoptotic bodies. Apoptosis occurs by either the extrinsic or intrinsic pathway. The extrinsic pathway is mediated by death receptors Fas, Tumor necrosis factor-alpha (TNF-α) receptor 1 (TNF-R1), and death receptors 4 and 5 (DR4/5) and their corresponding ligands FasL (Fas ligand), TNF-α, and TRAIL (TNF-related apoptosis-inducing ligand), which are all expressed in the liver. After a ligand binds its death receptor, a proteolytic

cascade results in activation of effector caspases and leads to cell death. The intrinsic pathway is triggered by DNA damage, oxidative stress, and toxins among other intracellular stress inducers, which causes mitochondrial damage and release of mitochondrial apoptotic factors such as cytochrome c and SMAC/DIABLO to trigger apoptosis. This pathway is regulated by proteins of the B-cell lymphoma-2 (Bcl-2) family. However, in some cell types such as hepatocytes, activation of the extrinsic apoptotic pathway is not sufficient to induce apoptosis. In hepatocytes, upon activation of the extrinsic pathway, caspase 8 cleaves the pro-apoptotic protein Bid into tBid (truncated bid), which translocates to the mitochondria and activates Bax or Bak. Bax or Bak is then inserted into the mitochondrial membrane, which results in permeabilization of the mitochondrial outer membrane and release of pro-apoptotic proteins from the mitochondrial intramembrane space into the cytosol and thus activates the intrinsic apoptotic pathway. Proteins released include proteins that promote further caspase activation (cytochrome c and SMAC/DIABLO) and proteins that translocate to the nucleus to degrade DNA (apoptosis inducing factor and endonuclease G) (Guicciardi, Malhi et al. 2013).

Necrosis in the liver is caused by drug-induced liver injury, such as acetaminophen overdose (further discussed in section 1.6) (Luedde, Kaplowitz et al. 2014) and ischemia/reperfusion-induced liver injury (Guicciardi, Malhi et al. 2013). Necrosis results from permeabilization of the cell membrane, loss of membrane potential and cell membrane rupture, which leads to release of cellular contents followed by an inflammatory response (Guicciardi, Malhi et al. 2013). Interestingly, mitochondrial damage also plays a critical role in either acetaminophen overdose or ischemia/reperfusion-induced necrosis. The lack of apoptosis in these conditions is likely due to the severe mitochondrial damage resulting in the rapid depletion of cellular ATP levels because ATP is required for caspase activation.

Mitochondria that are damaged produce ROS and release pro-apoptotic proteins or result in inflammation when necrotic, which amplifies cell death and injury. Therefore, it is

important to remove these damaged mitochondria to avoid hepatocellular death and injury in the liver (Mizushima, Levine et al. 2008). Mitophagy is protective against both apoptotic and necrotic cell death in the liver by removing damaged mitochondria. For example, mitophagy has been shown to be protective against apoptosis and injury induced by alcoholic-liver disease (further discussed in section 4) (Ding, Li et al. 2010, Lin, Zhang et al. 2013) and also against necrosis in acetaminophen-induced liver injury (further discussed in section 3) (Ni, Bockus et al. 2012, Ni, Williams et al. 2013, Lin, Wu et al. 2014, Saberi, Ybanez et al. 2014).

1.4.2. Mitophagy Maintains Mitochondrial Bioenergetics

Mitochondrial bioenergetics refers to a mitochondrion's capacity and efficiency for ATP production (Liesa and Shirihai 2013) and are regulated by substrate availability, cell energy requirements, and the overall quality and abundance of the mitochondria population (Hill, Benavides et al. 2012). Interestingly, mitochondrial bioenergetics and mitochondrial dynamics are linked. Cells in an environment rich with nutrients have fragmented mitochondria, while cells in a starvation environment have more elongated and fused mitochondria. Furthermore, these elongated mitochondria avoid degradation by mitophagy, once again emphasizing the importance of mitochondrial fragmentation for mitophagy induction as previously discussed in section 1.3.1 (Molina, Wikstrom et al. 2009, Gomes, Di Benedetto et al. 2011).

Removal of damaged mitochondria via mitophagy is crucial for preventing cell death and injury caused by mitochondrial uncoupling, which is described as a dysfunctional proton gradient in mitochondria that leads to uncoupling of oxidative phosphorylation and ATP production. Uncoupling causes damaged mitochondria and cell death by apoptosis (Hill, Benavides et al. 2012). Removing uncoupled mitochondria via mitophagy prevents spread of oxidative stress, cell death, and liver injury by maintaining a healthy population of mitochondria that can produce necessary energy efficiently for cell survival.

1.4.3 Mitophagy Maintains Fatty Acid Oxidation

Mitochondrial fatty acid oxidation (FAO) plays many important functions in the liver including providing substrates for gluconeogenesis, contribution of electrons to the respiratory chain for oxidative phosphorylation and ATP production, generation of acetyl-CoA for gluconeogenesis and ketogenesis (Kompare and Rizzo 2008), and degradation of lipids to prevent hepatocellular steatosis (Gao and Bataller 2011).

Fatty acids are a common source of energy and are often stored as non-toxic triaglycerols. They originate from several sources including *de novo* lipogenesis, triacylglycerol stores, and plasma non-esterified fatty acids released from adipose tissue (Nguyen, Leray et al. 2008). Long-chain fatty acids used for FAO are stored mainly in the adipose tissue as components of triglycerides or phospholipids. Lipases cause these triglycerides to release their fatty acids, which are then transported to the liver and muscle via the bloodstream. For use in FAO, these long-chain fatty acids must first be converted into acylcarnitine esters so they can be transported across the inner mitochondrial membrane into the mitochondrial matrix. The rate-limiting enzyme for shuttling these carnitines across the mitochondrial membrane is carnitine palmitoyltransferase I (CPT I), which is inhibited by malonyl-CoA in fed conditions. However, in fasting conditions, malonyl-CoA concentrations drop and CPTI is activated to begin FAO. Once in the mitochondria, fatty acids are processed by β-oxidation resulting in the release of acetyl-CoA, which can enter the TCA cycle, and production of FADH2 and NADH, which donate their electrons to the electron transport chain for ATP production (Kompare and Rizzo 2008, Nguyen, Leray et al. 2008).

Several liver diseases are exacerbated due to dysfunctional mitochondria and FAO such as fatty liver caused by alcoholic-liver disease (Beyoglu and Idle 2013) and drug-induced liver injury (Begriche, Massart et al. 2011). For example, β-oxidation is inhibited while fatty acid synthesis is upregulated in alcoholic liver disease, which results in an accumulation of lipids in

the liver and development of liver steatosis (Begriche, Massart et al. 2011, Gao and Bataller 2011). In addition, liver steatosis is induced by several different pharmaceuticals such as amiodarone, ibuprofen, acetaminophen, and tamoxifien, which inhibit enzymes needed for effective β-oxidation (Begriche, Massart et al. 2011). Consequences of reduced or inhibited β-oxidation include accumulation of fatty acids that are esterified and stored as triglycerides or an accumulation of fatty acids that remain in an un-esterified form. If accumulated in their un-esterified form, fatty acids can cause further mitochondrial damage and injury. In addition, faulty β-oxidation can impair energy output due to reduced production of ketone bodies and ATP (Begriche, Massart et al. 2011). Because mitophagy acts as an important regulator of mitochondrial homeostasis and helps to maintain a healthy population of mitochondria, it is likely that mitophagy helps defend against lipid accumulation in the liver by removing damaged mitochondria incapable of performing efficient β-oxidation.

1.4.4 Mitophagy and Mitochondrial Biogenesis Must be Balanced

Mitochondria have evolved several methods to adapt to cellular stress induced by drugs or disease including mitophagy activation to degrade damaged mitochondria and stimulation of mitochondrial biogenesis to replace damaged mitochondria removed by mitophagy. The number of mitochondria in cells is balanced by mitochondrial biogenesis and degradation by mitophagy. Changes in demand for mitochondrial function can shift the balance between these, but failing to restore the balance leads to mitochondrial dysfunction, cell death, and disease. For example, a decrease in mitochondria is associated with cell death, aging, Parkinson's disease, and liver disease. However, failure to remove mitochondria that are damaged leads to apoptosis and disease due to an accumulation of damaged mitochondria and overall mitochondrial dysfunction (Hill, Benavides et al. 2012, Zhu, Wang et al. 2013).

Mitochondrial biogenesis consists of *de novo* synthesis of mitochondrial components from other cellular precursors, formation of mitochondrial membranes, and division of currently existing mitochondria (Michel, Wanet et al. 2012). Mitochondrial biogenesis requires mitochondrial DNA transcription and translation, transcription and translation of nuclear DNA, and the assembly of complexes needed for oxidative phosphorylation. In addition, mitochondrial biogenesis requires protein import because most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol, and later transported into the mitochondria (Zhu, Wang et al. 2013, Palikaras and Tavernarakis 2014). Regulators of mitochondrial biogenesis include peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α), nuclear respiratory factors 1 and 2 (Nrf1/2), and mitochondrial transcription factor A (TFAM) and are activated by nutrient availability, hormones, and growth factors (Palikaras and Tavernarakis 2014).

There must be cross-talk between mitochondrial biogenesis and mitophagy pathways in order to provide a balanced population of healthy, functioning mitochondria. There are several pathways that simultaneously regulate both mitochondrial biogenesis and autophagy in order to maintain their balance including Parkin and AMPK. Parkin and AMPK promote both mitophagy and mitochondrial biogenesis simultaneously. The role of Parkin in mitochondrial biogenesis is further discussed in section 1.5.3. AMPK, a protein kinase, is activated in response to environmental stress or nutrient deprivation and activates mitochondrial biogenesis by either enhancing SIRT1 deacetylation of PGC-1α resulting in its nuclear translocation (Canto, Gerhart-Hines et al. 2009), or by enhancing PGC-1α activity through phosphorylation (Jager, Handschin et al. 2007, Scarpulla, Vega et al. 2012). While activating mitochondrial biogenesis, AMPK simultaneously upregulates mitophagy by either suppressing mTOR, which is a negative regulator of autophagy (Mihaylova and Shaw 2011), or by phosphorylating and activating ULK1/2 (Kim, Kundu et al. 2011) (discussed in section 1.2.3).

1.5 Roles of Parkin Independent of Mitophagy.

Parkin has several roles in maintenance of cellular homeostasis in addition to its role in promoting induction of mitophagy including removal of mitochondrial vesicles, prevention of mitochondrial spheroid formation, activation of mitochondrial biogenesis, lipid transport, and cell cycle regulation. The various roles of Parkin are summarized in **Figure 1.5**.

1.5.1 Removal of Mitochondrial Vesicles

Parkin and PINK1 stimulate the biosynthesis of mitochondria-derived vesicles (MDVs) under conditions of mitochondrial oxidative stress. Even though this pathway requires Parkin and PINK1, it is different from canonical mitophagy because it is stimulated by ROS production instead of mitochondrial depolarization. In addition, vesicles bud off of damaged mitochondria and are degraded in the lysosome along with their contents without involvement of the autophagy pathway (McLelland, Soubannier et al. 2014). These vesicles contain oxidized proteins (Soubannier, Rippstein et al. 2012), and they may regulate mitochondrial quality control using a mechanism faster than mitophagy to prevent complete mitochondrial depolarization while preserving mitochondrial function by selectively degrading damaged mitochondrial contents (Soubannier, Rippstein et al. 2012, McLelland, Soubannier et al. 2014).

1.5.2 Prevention of Mitochondrial Spheroid Formation

Mitochondrial spheroids are structurally unique mitochondria with a ring or cup-like morphology. They look similar to autophagosomes with the interior lumen being surrounded by mitochondrial membranes, but they have a small opening that connects the spheroid lumen to the cytosol, unlike autophagosomes (**Figure 1.6.5**). The significance of this opening is currently unclear. These spheroids can enwrap contents of the cytosol such as endoplasmic reticulum, lipid droplets, or other mitochondria. They are also positive for lysosome proteins and may have

some amount of degradation capacity, but it remains to be proven if they actually degrade contents within their lumen. Formation of mitochondrial spheroids requires the presence of ROS in addition to either MFN1 or MFN2, which allow for mitochondrial fusion. Deletion of either MFN1 or MFN2 inhibits spheroid formation, indicating that both fusion proteins are important for the formation of mitochondrial spheroids (Ding, Guo et al. 2012). Parkin can therefore inhibit formation of mitochondrial spheroids by inducing proteasomal degradation of MFN1/2 by ubiquitination, which was previously discussed in section 1.3.2. MEF cells, which have an undetectable Parkin expression level, formed mitochondrial spheroids after CCCP treatment. When Parkin was overexpressed in these cells, MFN1 and MFN2 were degraded and mitochondria underwent typical mitophagy instead of forming mitochondrial spheroids (Ding, Li et al. 2012). These data suggest that Parkin prevents mitochondrial spheroid formation in order for mitophagy to occur. Whether or not these spheroids represent a protective mechanism in the liver in addition to mitophagy requires further investigation (Ding, Guo et al. 2012, Ding, Li et al. 2012, Ni, Williams et al. 2013, Yin and Ding 2013).

1.5.3. Activation of Mitochondrial Biogenesis

Parkin is implicated in activation of mitochondrial biogenesis via regulation of Tfam (Kuroda, Mitsui et al. 2006, Rothfuss, Fischer et al. 2009) and Pgc-1α (The roles of Tfam and Pgc-1α in mitochondrial biogenesis were discussed in section 1.4.4). Parkin directly regulates Tfam in proliferating and in differentiated SH-SY5Y cells (human neuroblastoma cell line) along with mouse and human brain. Parkin regulates Tfam by binding it and enhancing its transcriptional activity, resulting in increased transcription and replication of mitochondria DNA (mtDNA) and suppression of mtDNA damage during oxidative stress. Parkin co-associates with Tfam and mtDNA (Kuroda, Mitsui et al. 2006, Rothfuss, Fischer et al. 2009). Parkin regulates Pgc-1α indirectly by ubiquitinating and inducing proteasomal degradation of PARIS (Parkin

Interacting Substrate), which is a transcriptional repressor of Pgc-1α. Degradation of PARIS by Parkin caused up-regulation of mitochondrial biogenesis in SH-SY5Y cells and in mouse and human brain, which protected against neurodegeneration (Shin, Ko et al. 2011). The role of Parkin in mediating mitochondrial biogenesis, in combination with mitophagy, in the brain is thought to be an important protective mechanism against Parkinson's disease by maintaining a healthy population of functional mitochondria. However, the role of Parkin in mediating mitochondrial biogenesis in the liver has not been studied.

1.5.4. Fat Transport

Parkin KO mice are smaller and gain weight slowly compared to WT mice (Palacino, Sagi et al. 2004), suggesting that Parkin may have some role in lipid metabolism. Parkin regulates fat uptake by stabilizing CD36 expression through monoubiquitination. CD36 is a transporter for long chain polyunsaturated fatty acids. When challenged with a high-fat diet, Parkin KO mice were protected against weight gain, insulin resistance, and steatosis of the liver. However, WT mice gained weight and developed liver steatosis and insulin resistance after high-fat diet feeding, which was concluded to be due to Parkin's role in stabilizing expression of CD36. In addition, fat uptake was shown to be affected by Parkin in the brain because fat uptake was increased in SH-SY5Y cells overexpressing Parkin and decreased in cells from patients with Parkin-mutant associated Parkinson's disease (Kim, Stevens et al. 2011). Parkin expression was also found to be increased in white adipose tissue after high-fat diet feeding (Kim, Stevens et al. 2011, Cummins, Holden et al. 2014), and Parkin stabilization of CD36 expression in adipocytes is important for fat accumulation during adipogenesis (Kim, Stevens et al. 2011). Interestingly, Parkin expression decreases in the brain substantia nigra in response to high-fat diet feeding, differing from *in vitro* results from Kim *et al*. This decrease in Parkin expression was associated with increased expression of PARIS and decreased Pgc-1α

(previously discussed in section 1.5.3), suggesting that consumption of a high fat diet may increase vulnerability to Parkinson's disease by inhibiting mitochondrial biogenesis (Khang, Park et al. 2015). Nevertheless, all of these findings suggest that Parkin has a role in regulating lipid metabolism.

1.5.5. Cell Cycle Regulation and Tumor Suppression

Parkin is a tumor suppressor, and it is one of the most frequently deleted genes in human cancers. The *Park2* gene was found to be mutated in many different types of cancer including cervical, colorectal, gastric, glioblastoma, and lung adenocarcinoma among others. Cancer-causing mutations are likely due to inactivation of Parkin activity. Interestingly, cancer-associated mutations for *Park2* occur within the same regions as mutations causing Parkinson's disease, but human patients with *Park2* mutations do not have greater incidences of cancer with the exception of one family containing eight people with lung cancer that were recently found to have germline *Park2* mutations (Veeriah, Morris et al. 2010, Alcalay, Clark et al. 2012, Xu, Lin et al. 2014).

Copy number loss of *Park2* is associated with breast, brain, lung, ovarian, and pancreatic cancers, and the *Park2* gene is also suggested to be inactivated in leukemia and colorectal cancer by promoter hypermethylation (Xu, Lin et al. 2014). In addition, decreased expression of Parkin is associated with liver (Wang, Denison et al. 2004), bladder, lung, breast, brain, and thyroid cancers among others (Xu, Lin et al. 2014), and restoration of Parkin expression in breast cancer cell lines (Tay, Yeo et al. 2010) and glioma cells (Veeriah, Taylor et al. 2010) slowed cellular proliferation. Furthermore, Parkin expression is reduced in human hepatocellular carcinoma primary tumors and cell lines (Wang, Denison et al. 2004), and Parkin KO mice develop liver tumors at 18 months of age (Fujiwara, Marusawa et al. 2008).

The involvement of Parkin in tumorigenesis is likely due to its role in cell cycle regulation, its transcriptional regulation by p53, and its maintenance of mitochondrial homeostasis. Parkin ubiquitinates and mediates proteasomal degradation of Cyclin E and Cyclin D1 *in vitro*, and Parkin loss upregulates expression of these proteins leading to progression through the cell cycle and increased proliferation (Veeriah, Morris et al. 2010, Gong, Zack et al. 2014). Cyclin D1 is complexed with Cyclin-dependent kinases (CDK) CDK4 and CDK6 and is required for transition from G1 phase to the DNA replication phase (S phase) of the cell cycle, which occurs through Cyclin D1/CDK4/CDK6-mediated phosphorylation and inactivation of the cell cycle inhibitor Retinoblastoma protein. This leads to the release of transcription factors, such as E2F-1, which induce expression of genes needed for advancement through the cell cycle, such as Cyclin E and Cyclin A. Cyclin E forms a complex with CDK2 and is also important for progression from G1 into S phase by phosphorylating Retinoblastoma protein, similar to Cyclin D1, in addition to phosphorylating the cell-cycle inhibitor p27. Cyclin A participates in regulation of multiple steps of the cell cycle via interaction with CDK1 and CDK2, which are associated with the G2/ M phase (mitosis/cell division) transition or S phase, respectively (Vermeulen, Van Bockstaele et al. 2003). In addition, Parkin regulates Beta-catenin protein levels in the brain through ubiquitination and degradation (Rawal, Corti et al. 2009). Beta-catenin is well known to induce expression of genes necessary for cell-cycle progression and proliferation including Cyclin D1, and it is commonly mutated in cancer (Nejak-Bowen and Monga 2011). However, the role of Parkin in regulation of beta-catenin in the liver is unknown.

Parkin expression is also transcriptionally regulated by p53, which is a well-known anti-cancer gene that arrests cell cycle progression and initiates DNA repair and / or apoptosis upon DNA damage (Zhu, Lu et al. 2014). Mutations and down-regulation of p53 are associated with many types of cancer, and loss of p53 can also further cancer progression by decreasing Parkin

expression in addition to loss of its other anti-tumorigenic functions (Zhang, Lin et al. 2011, Viotti, Duplan et al. 2014).

Finally, Parkin's mediation of mitophagy (section 1.3.2) and mitochondrial biogenesis (section 1.5.3) likely play anti-tumorigenic roles by maintaining a healthy population of mitochondria because damaged mitochondria are well known to be involved in cancer progression (Gogvadze, Orrenius et al. 2008).

Figure 1.5

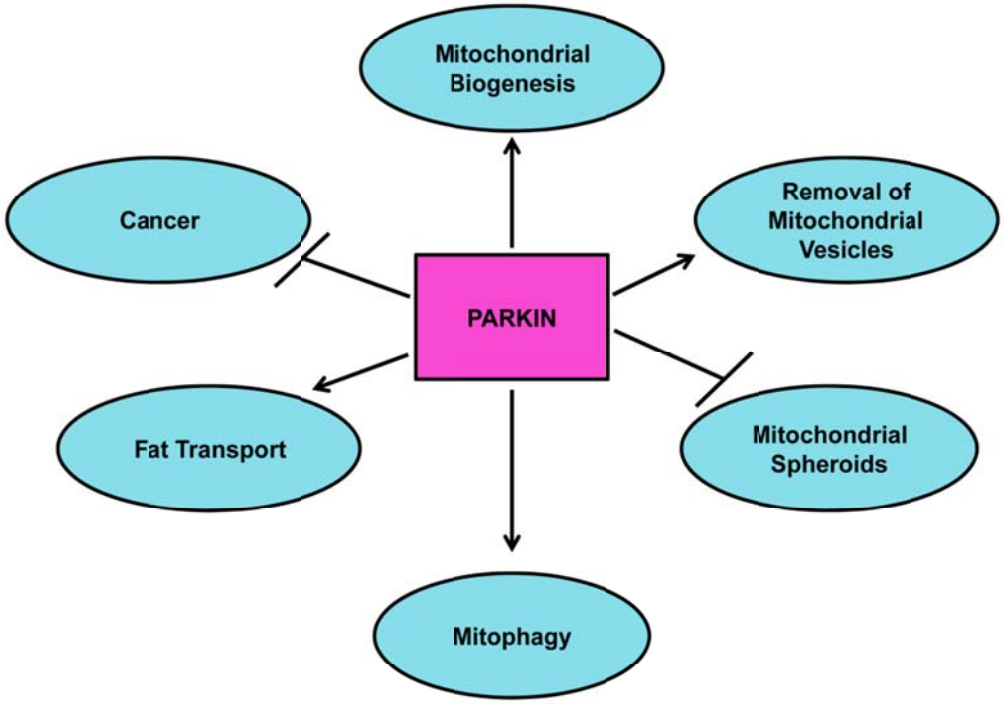

Figure 1.5: Various Roles of Parkin. Parkin has been shown to activate mitophagy and mitochondrial biogenesis. Parkin has also been shown to have a role in fat uptake and in removal of mitochondrial vesicles. Parkin inhibits formation of mitochondrial spheroids, and Parkin is a tumor suppressor.

1.6 Acetaminophen

1.6.1. Overview of Acetaminophen (APAP)

Acetaminophen (APAP) is a widely used drug with analgesic and antipyretic properties, but its therapeutic mechanism of action is currently unknown. APAP can be taken orally, intravenously, or rectally, and it can be taken individually or in combination with other drugs, such as over the counter cold and flu medications or prescribed pain medications like Percocet®. APAP is the most commonly used drug in the United States (Kaufman, Kelly et al. 2002) with approximately 50 million adults taking some formulation of APAP each week (Hinson, Roberts et al. 2010). APAP overdose is the most common cause of acute liver failure in the United States (Larson, Polson et al. 2005), and it is responsible for approximately 26,000 hospitalizations per year with nearly 500 cases resulting in death (Nourjah, Ahmad et al. 2006). APAP is responsible for approximately 40% of all cases of acute liver failure in the United States (Larson, Polson et al. 2005, Blieden, Paramore et al. 2014), and care for these cases costs an estimated 87 million dollars per year (Bond and Novak 1995). APAP is considered safe when taken at the therapeutic doses (350-600 mg per mouth every 4 to 6 hours for a daily maximum of 4 grams for an adult) (Schilling, Corey et al. 2010), but it can cause severe liver damage and even death when taken in greater amounts. APAP overdose often happens during suicide attempt or by accident when several medications containing APAP are taken in combination.

1.6.2 APAP Metabolism and Mechanism of Toxicity

APAP is absorbed from the duodenum and metabolized in the liver after ingestion. The majority of APAP taken at a therapeutic dose is eliminated during phase II metabolism by glucoronidation or sulfation, which makes APAP more water soluble and allows for its excretion in the urine by the kidney. Glucoronidation, which is catalyzed by UDP-glucuronosyl transferases (UGT), is responsible for approximately 60% of APAP conjugation and excretion.

Sulfation, which is catalyzed by sulfotransferases (SULT), accounts for approximately 30% of APAP conjugation and excretion. The remaining approximate 10% of APAP is converted to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) during phase I metabolism by cytochrome p450s (Hinson, Roberts et al. 2010, McGill and Jaeschke 2013). NAPQI is an electrophile that reacts with proteins by binding to their sulfhydryl groups (Jollow, Mitchell et al. 1973, Corcoran, Mitchell et al. 1980, Streeter, Dahlin et al. 1984). Even though NAPQI is a reactive metabolite, it is detoxified and excreted once it is bound to glutathione (GSH) (Mitchell, Jollow et al. 1973). The cytochrome p450s CYP2E1, CYP1A2, CYP2D6, and CYP3A4 have all been shown to have a role in metabolizing APAP to NAPQI (Raucy, Lasker et al. 1989, Patten, Thomas et al. 1993, Snawder, Roe et al. 1994, Dong, Haining et al. 2000), but CYP2E1 is the most prominent cytochrome p450 responsible for this (Cheung, Yu et al. 2005, McGill and Jaeschke 2013).

When taken at therapeutic doses, APAP is considered safe because the reactive metabolite NAPQI is detoxified by GSH and excreted. However, an abundance of NAPQI causes GSH depletion in APAP overdose situations, leaving NAPQI free to bind to cysteine residues of proteins, forming protein adducts (Streeter, Dahlin et al. 1984, Hinson, Reid et al. 2004). APAP protein adducts in hepatocytes lead to their necrosis and subsequent liver injury (Jollow, Mitchell et al. 1973, Bartolone, Cohen et al. 1989, Roberts, Bucci et al. 1991). Most adducted proteins are cytosolic, but several mitochondrial proteins also contain adducts (Qiu, Benet et al. 1998). Known adducted proteins include glutamine synthase, glutamate dehydrogenase, aldehyde dehydrogenase, glutathione peroxidase, proteasome subunit 5, and ATP synthase α subunit. However, the exact role of APAP-adduction of these proteins in the development of APAP-induced hepatotoxicity is not yet known (Hinson, Roberts et al. 2010).

Formation of mitochondrial protein adducts is crucial for initiation of APAP toxicity because mitochondrial protein adducts can lead to mitochondrial damage, production of reactive

oxygen species (ROS), and subsequent cell death (Pumford, Roberts et al. 1990, Jaeschke, McGill et al. 2012). APAP overdose promotes mitochondrial translocation of Bax, which together with Bak, forms pores in the outer mitochondrial membrane. Pore formation leads to release of cytochrome c in addition to endonuclease G and apoptosis inducing factor, which cause DNA fragmentation and eventual necrosis (Bajt, Farhood et al. 2008). APAP overdose also causes oxidative stress leading to mitochondrial transition pore (MPT) opening, which causes mitochondrial depolarization, mitochondrial swelling, and decreased respiration and ATP synthesis. MPT opening further exacerbates oxidative stress and mitochondrial damage, causing hepatocytes to undergo necrosis leading to liver injury (Kon, Kim et al. 2004, Masubuchi, Suda et al. 2005).

APAP-induced oxidative stress causes activation of c-Jun N-terminal kinase (JNK), which results in its mitochondrial translocation, increased oxidative stress, and exacerbation of APAP-induced liver injury (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007, Hanawa, Shinohara et al. 2008, Saito, Lemasters et al. 2010). JNK is activated by phosphorylation during the early phase of APAP-induced liver injury (2 to 4 hours after APAP administration in mice) (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007), and inhibition of JNK activation in mice and mouse hepatocytes by a one hour pretreatment with the JNK inhibitor SP600125 was protective against APAP-induced liver injury (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007). Post-treatment with the JNK inhibitor also protected against APAP-induced liver injury at 5 hours, but lost its protective effect by 8 hours post-treatment (Henderson, Pollock et al. 2007). Even though use of a JNK inhibitor, which blocks both JNK1 and JNK2, was protective against APAP-induced liver injury and necrosis, JNK1 knockout mice were not protected against APAP-induced hepatotoxicity (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007). The role of JNK2 is controversial because JNK2 KO mice have been found to be both partially protected (Gunawan, Liu et al. 2006) and not protected (Henderson, Pollock et al. 2007) against

APAP-induced liver injury. These data suggest that simultaneous loss of both JNK1 and JNK2 activation may be required for protection against APAP-induced liver injury, but this is still under debate. In addition, it was suggested that APAP-induced activation of JNK occurs through oxidative-stress mediated activation of apoptosis-signal-relating kinase 1 (ASK1), which is an upstream mediator of JNK phosphorylation. A 30 minute pretreatment with an inhibitor of ASK1 (GS-459679) protected against APAP-induced liver injury by inhibiting JNK activation, translocation of JNK to mitochondria, and reducing oxidative stress. A 1.5 hour post-treatment with the ASK1 inhibitor also protected against APAP-induced liver injury, but this protection was lost by 3 hours post-treatment (Xie, Williams et al. 2013).

In summary, APAP-induced hepatotoxicity occurs by conversion of APAP to the reactive metabolite NAPQI, which is bound by GSH and causes GSH depletion upon APAP overdose. NAPQI then binds to mitochondrial proteins, which initiates mitochondrial damage and oxidative stress leading to MPT pore opening. MPT pore opening causes more oxidative stress and mitochondrial damage and leads to reduced respiration and decreased production of ATP. Oxidative stress also leads to JNK activation and JNK mitochondrial translocation, which exacerbates mitochondrial damage and oxidative stress, leading to hepatocellular necrosis and subsequent liver injury. There are also several factors that affect APAP metabolism, causing greater risk of APAP overdose and liver injury. For example, malnutrition and pre-existing liver disease cause a reduction in GSH levels, resulting in impaired detoxification of NAPQI. CYP2E1-inducing drugs or induction of CYP2E1 via chronic alcohol abuse can also cause increased risk of APAP overdose by resulting in greater NAPQI production after APAP use (Blieden, Paramore et al. 2014).

1.6.3 Mechanisms of Repair in APAP-induced Liver Injury

The most important mechanism of repair in APAP-induced liver injury is hepatocellular proliferation, which allows for replacement of damaged hepatocytes. Liver regeneration begins approximately 24 hours after APAP treatment in mice. TNFα, IL-6, vascular endothelial growth factor (VEGF), and β-Catenin each contribute to initiation of hepatocyte proliferation after APAP-induced liver injury (Chiu, Gardner et al. 2003, James, Lamps et al. 2003, Donahower, McCullough et al. 2006, Kato, Ito et al. 2011, Bhushan, Walesky et al. 2014). TNFα is a well-known activator of the NF-κB pathway, which results in transcription of many proliferative genes, such as Cyclin D1 (Guttridge, Albanese et al. 1999). TNFα and NF-κB along with the cell cycle proteins Cyclin D1 and Cyclin A (Cyclin D1 and Cyclin A were previously discussed in section 1.5.5) are induced after APAP treatment in mice and mediate liver regeneration after APAP treatment (Chiu, Gardner et al. 2003, Bhushan, Walesky et al. 2014). Mice lacking the TNFα receptor 1 (TNFR1 KO) exhibit increased liver injury along with decreased Cyclin D1, Cyclin A, and hepatocyte proliferation, suggesting that TNFα is an important mediator of liver regeneration after APAP-induced liver injury (Chiu, Gardner et al. 2003).

IL-6 is also involved in liver regeneration by activating the transcription factor signal-transducer and activator of transcription 3 (STAT-3), which induces expression of genes involved in cellular proliferation such as Cyclin D1 among many others (Wang, Lafdil et al. 2011, Carpenter and Lo 2014). Loss of IL-6 in mice did not result in increased APAP-induced liver injury compared to WT mice, but IL-6 KO mice had decreased proliferating hepatocytes after APAP treatment. In addition, pretreatment of IL-6 KO mice with IL-6 reduced liver injury and increased proliferation levels after APAP treatment, suggesting IL-6 is a mediator of liver regeneration after APAP overdose (James, Lamps et al. 2003). However, this is controversial because IL-6 was also shown to be down-regulated after APAP treatment. In addition, IL-6

expression and STAT-3 activation were upregulated in mouse livers that were unable to regenerate (Kato, Ito et al. 2011, Bhushan, Walesky et al. 2014).

VEGF, which is an important regulator of angiogenesis, is also upregulated after APAP treatment along with its receptors VEGFR1, VEGFR2, and VEGFR3 (Donahower, McCullough et al. 2006, Kato, Ito et al. 2011). Treatment with a VEGF receptor inhibitor (Semaxanib/ SU5416) resulted in slightly increased liver injury 48 hours after APAP treatment, but not in the pre-regenerative phase at 6 and 24 hours. The VEGFR inhibitor also caused reduced hepatocyte proliferation (Donahower, McCullough et al. 2006). Use of VEGFR1 Tyrosine-Kinase (TK) KO mice showed that VEGF is an important mediator of liver regeneration after APAP treatment. VEGFR1-TK KO mice had increased liver injury 48 hours after APAP treatment compared to WT mice, but not in the early phases after APAP treatment. In addition, these mice had decreased proliferation along with increased hemorrhage and mortality after APAP-treatment compared to WT mice (Kato, Ito et al. 2011). These data suggest that VEGF and angiogenesis have a role in liver regeneration after APAP overdose.

The Wnt/β-catenin pathway also contributes to liver regeneration after APAP overdose by inducing expression of Cyclin D1. The Wnt/β-catenin pathway was activated after APAP treatment, and mice overexpressing β-catenin had increased liver regeneration, but similar liver injury, compared to WT mice (Bhushan, Walesky et al. 2014). Bhushan *et al.* also demonstrated that liver regeneration following APAP-induced liver injury is dependent on a dose-response. A lower 300 mg/kg dose of APAP resulted in liver regeneration while a higher 600 mg/kg dose inhibited regeneration, leading to sustained liver injury and mortality in mice. Therefore, in addition to induction of pathways mentioned in this section, the ability of the liver to regenerate after APAP overdose depends on if the APAP amount consumed is less than the threshold dose for regeneration (Bhushan, Walesky et al. 2014).

1.6.4. Clinical signs of APAP Toxicity and Treatment Options

During APAP overdose, patients experience nausea, vomiting, and abdominal pain within 2-3 hours after ingestion, which can progress to jaundice and encephalopathy within several days (Blieden, Paramore et al. 2014). Liver damage and dysfunction occur within 24 hours and peak between 3 and 4 days after overdose (Boyer and Rouff 1971). Clinical signs of APAP overdose include increased serum levels of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and signs of liver dysfunction such as hyperbilirubinemia and increased prothrombin time (Hinson, Roberts et al. 2010). Levels of 3-cysteine-APAP adducts in serum also correlate with patient liver toxicity (Muldrew, James et al. 2002). However, adduct levels were also increased in plasma in the absence of liver injury, suggesting that this parameter may not directly correlate with liver injury (McGill, Lebofsky et al. 2013). The half-life of APAP, usually 1.5 to 3 hours in the blood (Nelson and Morioka 1963), also increases after overdose and correlates with the extent of liver damage (Schiodt, Ott et al. 2002).

N-acetyl-cysteine (NAC) is the only treatment option for APAP overdose other than liver transplantation (Lancaster, Hiatt et al. 2015). In 1973, Mitchell et al. administered cysteine to mice, which reduced their APAP-induced liver injury (Mitchell, Jollow et al. 1973). These experiments eventually led to the development of NAC (Mucomyst®) for treatment of APAP hepatotoxicity in 1976 (Piperno and Berssenbruegge 1976, Peterson and Rumack 1977). When administered during APAP metabolism, NAC is a very effective therapeutic option in cases of APAP overdose. NAC functions by maintaining a supply of GSH because it increases GSH synthesis and it also directly conjugates to NAPQI, leading to its detoxification and prevention of liver injury. However, if administered too late after APAP metabolism, NAC is ineffective because it can no longer reduce the amount of reactive metabolite available to initiate liver injury (Corcoran, Racz et al. 1985, Corcoran, Todd et al. 1985, Corcoran and Wong 1986). For example, patients treated with NAC within 10 hours after overdose had 4-fold decreased liver

injury compared to patients treated between 10 and 16 hours and 9-fold decreased liver injury compared to patients treated between 16 and 24 hours (Rumack, Peterson et al. 1981). Even though JNK inhibitors and ASK1 inhibitors (discussed in section 1.6.2) are protective against APAP-induced liver injury in mice, they also must be given very early after APAP overdose to be effective. Therefore, therapeutic use of these inhibitors would likely not produce better outcomes than NAC.

1.6.5. Autophagy Protects against APAP-induced Liver Injury by Removing Damaged Mitochondria

Autophagy was extensively discussed in section 1.2. Our lab demonstrated that autophagy is induced after APAP treatment in both mouse livers and primary mouse hepatocytes, and this autophagy induction is likely due to APAP-induced suppression of mTOR. Pharmacological induction of autophagy via rapamycin protected against APAP-induced hepatocellular necrosis in primary hepatocytes, while autophagy inhibition by 3-MA and CQ exacerbated necrotic cell death. In addition, a 3 hour post-treatment with rapamycin also protected against APAP-induced necrosis in primary hepatocytes. Furthermore, rapamycin treatment protected against APAP-induced liver injury, and inhibition of autophagy by chloroquine exacerbated APAP-induced liver injury, *in vivo*. We showed that protection against APAP-induced liver injury by autophagy is likely through selective removal of mitochondria by mitophagy (mitophagy was discussed in section 1.3) because many autophagosomes contained mitochondria after APAP treatment, and mitochondrial proteins were degraded in primary hepatocytes after APAP treatment (Ni, Bockus et al. 2012). However, the mechanism for induction of mitophagy in the liver after APAP overdose is unknown.

We also demonstrated that APAP induces zonated induction of mitophagy in the liver. The liver is divided into periportal, intermediate, and centrilobular zones. These zones have

differences in CYP450 enzyme expression and GSH levels with centrilobular hepatocytes having the highest expression of CYP450 enzymes and the lowest GSH levels (Lindros 1997, Jungermann and Kietzmann 2000). APAP is mainly metabolized in the centrilobular zone where expression of CYP450s is highest and GSH is the lowest, which causes the majority of hepatocellular necrosis to occur near the central vein (Jaeschke, Gores et al. 2002). Treatment of GFP-LC3 transgenic mice with APAP increased the formation of GFP-LC3 positive autophagosomes, which were also localized mainly to the centrilobular zone in the liver (Ni, Bockus et al. 2012). Autophagosomes likely localize to this particular area of the liver in order to degrade mitochondria damaged by APAP adduct formation via mitophagy.

We characterized APAP-induced liver injury into four zones. The four zones include hepatocellular necrosis, mitochondrial spheroid formation, mitophagy, and mitochondrial proliferation. Zone 1 contains necrotic hepatocytes. Zone 2 contains mitochondrial spheroids (discussed in section 1.5.2), which may protect against the spread of hepatocellular necrosis induced by APAP. However, the role of mitochondrial spheroid formation in protection against APAP-induced liver injury is not clear. Zone 3, which is adjacent to Zone 2, has normally structured autophagosomes containing mitochondria and is termed the “autophagy active area”. This zone has minimally damaged mitochondria after APAP treatment due to containing less CYP450s and more GSH, so the hepatocytes can adapt and use mitophagy to selectively degrade damaged mitochondria to prevent the spread of cellular necrosis. Zone 4, the outermost area surrounding Zone 3, contains proliferating hepatocytes. This area has very little damage from APAP overdose. Therefore, it does not have a need for mitophagy induction. In contrast, mitochondria in this area must proliferate in order to provide energy needed for hepatocellular proliferation and liver regeneration to repair/replace damaged hepatocytes induced by APAP (**Figure 1.6.5**) (Ni, Williams et al. 2013).

Figure 1.6.5

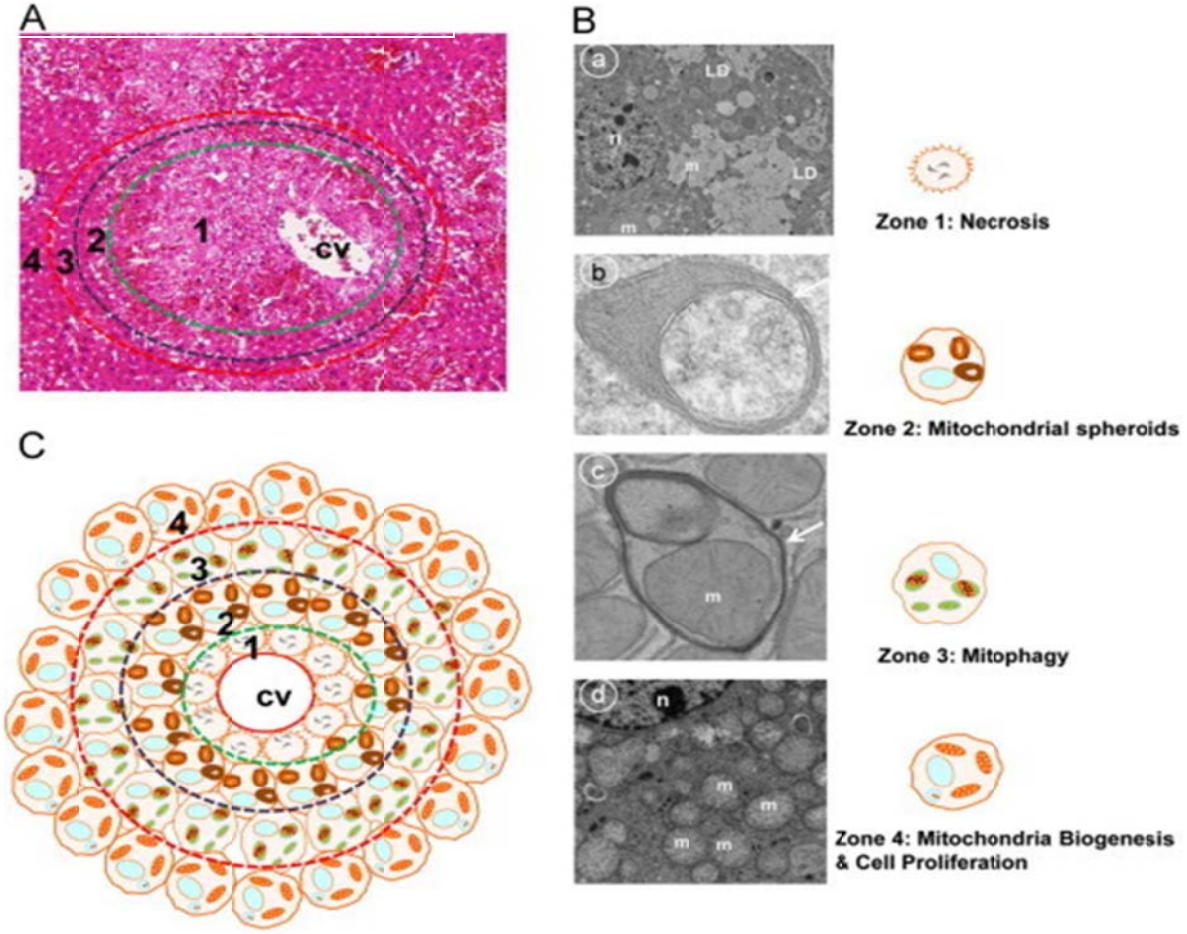

Figure 1.6.5: Distinctive zonated changes for necrosis, mitochondrial spheroids, mitophagy/autophagy and mitochondrial biogenesis/hepatocyte proliferation in APAP-induced liver injury. (A) Typical histological changes of APAP-induced centrilobular necrosis. Male C57BL/6 mice were treated with APAP (500 mg kg, i.p.) for 6 h and liver tissues were processed for H & E staining. Necrotic areas were mainly detected around the central vein (green circled area) and adjacent areas appeared to be normal unaffected hepatocytes. (B) Distinctive zonated morphological changes detected by electron microscopy analysis in APAP-induced mouse liver. Four distinctive zonated morphological changes are: Zone 1-necrosis (panel a) where cells display swollen mitochondria, condensed nuclei and accumulated lipid droplets (LD); Zone 2-mitochondrial spheroids (panel b) where mitochondria undergo structural remodeling with squeezed matrix and form a lumen that can enwrap cytosol, endoplasmic reticulum and even another mitochondria (an arrow denotes the squeezed matrix of a mitochondrial spheroid); Zone 3-mitophagy (panel c) where double membrane autophagosomes envelop mitochondria (an arrow denotes a typical autophagosome); Zone 4-mitochondrial biogenesis and cell proliferation (panel d) where hepatocytes have an increased number of mitochondria and PCNA positive cells. n: nucleus; m: mitochondria; LD: lipid droplet. Reproduced with permission from (Ni, Williams et al. 2013).

1.7. Alcoholic Liver Disease

Alcohol consumption and abuse are major causes of chronic liver disease, which is a significant health problem in the United States and around the world. Alcoholic liver disease (ALD) is a substantial problem that is caused by heavy alcohol consumption in addition to other environmental and genetic factors. Significant research progress has been made for understanding ALD pathogenesis, but a universal treatment to cure ALD is still lacking.

1.7.1. Metabolism, Pathogenesis, and Risk Factors for ALD

Alcohol metabolism occurs mainly in the liver, and the predominant pathway is mediated by alcohol dehydrogenase (ADH) in the cytosol, which metabolizes alcohol into acetaldehyde. Alcohol can also be oxidized by CYP2E1 in microsomes to acetaldehyde. In addition, catalase can oxidize alcohol to acetaldehyde in peroxisomes, but this is not a common pathway. Acetaldehyde is a reactive metabolite of alcohol that reacts with thiol and amino groups on proteins forming acetaldehyde-adducts, causing inhibition of protein function and tissue injury. Acetaldehyde is further metabolized into acetate by aldehyde dehydrogenase (ALDH1 or ALDH2), which is a harmless metabolite that can further be metabolized to acetate and acetyl-CoA for participation in metabolic pathways (Cederbaum 2012). Alcohol metabolism by ADH and ALDH require reduction of NAD^+ to NADH. Therefore, oxidative alcohol metabolism results in a decreased NAD^+ / NADH ratio and oxidative stress, leading to mitochondrial damage, down-regulation of metabolic pathways that require NAD+ as a co-enzyme, and liver injury (Cederbaum, Lu et al. 2009).

The pathogenesis of ALD in humans is characterized by steatosis (mild stage), which is an accumulation of fat in hepatocytes. In most heavy alcohol consumers, steatosis is caused by inhibiting fatty acid oxidation while increasing uptake of fat into the liver along with fatty acid and triglyceride synthesis. Steatosis is reversible with a few weeks of abstinence from alcohol, but

approximately 8-20% of heavy drinkers with steatosis can further develop steatohepatitis (moderate stage) and fibrosis and cirrhosis (advanced stage), and some (3-10%) eventually develop hepatocellular carcinoma (HCC) with prolonged alcohol abuse. Alcoholic steatohepatitis is characterized by hepatic inflammation and injury in addition to steatosis and also includes fibrotic and cirrhotic disease states. Steatohepatitis can also be reversible depending on if liver cirrhosis has developed. However, once cirrhosis has developed, less than 10% of patients can reverse their liver disease with abstinence. Most patients that stop using alcohol once cirrhosis has developed have a survival time of approximately 5 years after diagnosis (Teli, Day et al. 1995, Diehl 2002, Altamirano and Bataller 2011). Approximately 20% of all cases of liver cirrhosis in the United States are related to alcohol abuse (Singal, Kamath et al. 2013), and approximately 5 million people in the United States are thought to have alcohol-induced steatohepatitis (Basra and Anand 2011). Cirrhosis is the end stage of ALD, and it is characterized by massive fibrosis and the presence of regenerative nodules in the liver in addition to loss of liver function. Fibrosis is an exacerbated wound-healing response induced by liver injury that is characterized by an accumulation of extracellular matrix proteins, such as collagen, which are produced predominantly by hepatic stellate cells. Continuous activation of this wound healing response leads to cirrhosis of the liver, which can eventually progress to HCC (Teli, Day et al. 1995, Diehl 2002, Altamirano and Bataller 2011, Streba, Vere et al. 2014).

Most heavy alcohol consumers do not progress beyond steatosis of the liver, which suggests that other factors contribute to progression of ALD in addition to heavy alcohol consumption. There have been several factors shown to contribute to progression and severity of ALD in humans including race, sex, and comorbidities like obesity or hepatitis C virus. Genetic polymorphisms and epigenetic modifications also contribute to ALD progression. In addition, drinking patterns have been shown to have a role in ALD progression.

Two of the most important factors in susceptibility to ALD progression are race and sex. African-Americans and Hispanics are more likely to progress to alcohol-induced cirrhosis than Caucasians (Stinson, Grant et al. 2001, Stewart 2002). In addition, women are more likely to progress to ALD compared to men after alcohol consumption (Sato, Lindros et al. 2001).

Comorbidity with other diseases, such as obesity or hepatitis C virus (HCV), in addition to lifestyle factors also contribute to ALD progression. Obesity and metabolic syndrome have a synergistic effect on alcohol-induced liver injury (Naveau, Giraud et al. 1997, Chiang and McCullough 2014), and obesity is associated with an increased mortality rate in ALD patients (Stepanova, Rafiq et al. 2010). HCV is also associated with severity of ALD (Degos 1999, Monto, Patel et al. 2004), and the risk of developing cirrhosis increases in HCV patients that are heavy alcohol consumers (Harris, Gonin et al. 2001). Smoking also contributes to ALD severity and progression to liver cirrhosis (Corrao, Lepore et al. 1994, Pessione, Ramond et al. 2003, Altamirano and Bataller 2010). In contrast, drinking coffee protects against ALD severity and development of alcohol-induced liver cirrhosis (Corrao, Lepore et al. 1994, Stroffolini, Cotticelli et al. 2010).

Genetic polymorphisms and epigenetics also contribute to ALD progression and severity. For example, several polymorphisms of genes necessary for alcohol metabolism have been identified including polymorphisms in ADH2 and ADH3, ALDH2, and in the CYP2E1 promoter (Monzoni, Masutti et al. 2001, Zintzaras, Stefanidis et al. 2006). In addition to genetic polymorphisms, epigenetics also contributes to ALD progression. Alcohol influences epigenetics and histone modification in the GI tract and liver, which may increase progression and severity of ALD. For example, alcohol alters expression of ADH due to histone modification. More critically, epigenetic changes induced by alcohol consumption may be transmitted to offspring, which could affect their development (Shukla and Lim 2013).

Finally, the amount and duration of alcohol use in addition to the type of alcohol consumed and the pattern of alcohol consumption all have a role in progression of ALD. Women that drink more than 40 grams of alcohol per day and men that drink more than 80 grams per day were shown to be more likely to develop ALD, but some developed ALD by consuming less amounts of alcohol. In addition, wine drinkers were shown to be less likely than beer or liquor drinkers to develop alcoholic cirrhosis, but this is controversial. Heavy daily drinking was also shown to increase the risk for developing ALD in comparison to weekly binge drinking (Streba, Vere et al. 2014).

1.7.2 Clinical Signs and Treatment Options for ALD

Early stages of ALD, such as steatosis, are usually asymptomatic. Once patients progress to alcoholic steatohepatitis, most will have hepatomegaly and pain. Patients may also have jaundice, fever, encephalopathy, or splenomegaly. However, some steatohepatitis patients are asymptomatic. Patients that have progressed to liver cirrhosis can also present without symptoms, but most will have portal hypertension and hepatocellular dysfunction, which will cause them to be jaundiced and have ascites, hepatic encephalopathy, and bleeding in the gastrointestinal tract (Diehl 2002).

There are no specific clinical tests for diagnosis of ALD. ALD can cause elevation of ALT and AST, and AST levels are normally two to three times higher than ALT levels in ALD. Increased serum alkaline phosphatase levels along with decreased potassium, magnesium, and phosphate levels may indicate ALD. Patients may also have low levels of serum albumin, prolonged thrombin time, and elevated bilirubin levels (Diehl 2002, Streba, Vere et al. 2014). There may also be increases in serum inflammatory cytokines in steatohepatitis including TNFα, IL-6, and IL-8 (McClain, Hill et al. 1993). However, these serum markers are present in many different types of liver disease and do not specifically indicate ALD, so other liver diseases or

causes of liver injury must be ruled out before ALD can be diagnosed. Knowledge of a history of alcohol abuse is also helpful in combination with these disease markers for diagnosis. Imaging studies, such as sonography, may be used to visualize liver steatosis and ascites. Liver biopsy is the most reliable test for diagnosing ALD, but it is also the most invasive (Diehl 2002, Streba, Vere et al. 2014).

The only therapeutic options for ALD patients are abstinence from alcohol or liver transplant. Abstaining from alcohol has been shown to reverse ALD in early disease states and can decrease mortality rates even in late-stage cirrhosis (De, Gangopadhyay et al. 2009). Corticosteroids, such as prednisolone, reduced mortality by 37% in alcoholic steatohepatitis patients, but they are only effective in patients with hepatic encephalopathy (Imperiale and McCullough 1990). The TNFα inhibitor pentoxifylline improved survival rates in patients with alcoholic steatohepatitis. Pentoxifylline has a lower risk of gastrointestinal bleeding than corticosteroids, which makes it a better treatment option. Pentoxifylline also had a lower mortality rate compared to prednisolone treatment in one clinical trial. However, there were no differences in 6-month survival rates in patients that were given pentoxifylline in combination with prednisolone versus patients given prednisolone only (De, Gangopadhyay et al. 2009, Mathurin, Louvet et al. 2013). Liver transplant is the best option for decreasing mortality in ALD. However, the option for liver transplantation is only given to those with severe disease and / or to patients who have been sober for at least 6 months (Diehl 2002).

1.7.3 Role of Autophagy in ALD

Our lab and others recently demonstrated that alcohol consumption activates autophagy to selectively remove excess lipid droplets and damaged mitochondria, which attenuates alcohol-induced steatosis and liver injury in mice (Ding, Li et al. 2010, Ding, Manley et al. 2011, Dolganiuc, Thomes et al. 2012, Lin, Zhang et al. 2013). However, there is also evidence to

suggest that alcohol consumption may suppress autophagy, particularly in chronic alcohol consumption conditions (Donohue 2009, Thomes, Ehlers et al. 2013). Several possibilities could explain these discrepancies including animal models used to assess ALD, assays used to determine autophagy, and the limitation of steady-state assessment of autophagy using only one time point *in vivo*. For example, it is now well known that use of LC3-II levels to monitor autophagy is troublesome because LC3-II itself is degraded in autolysosomes during autophagy. For this reason, autophagic flux assays (discussed in section 1.2.5) are now mandatory for assessing autophagy (Klionsky, Abdalla et al. 2012), and autophagy flux was not always evaluated when determining the effect of ethanol on autophagy. Autophagy is also a dynamic process, and autophagic activity can fluctuate during experimental conditions over time (Ni, Bockus et al. 2011). Moreover, autophagy activity can be influenced by circadian rhythm (Ma, Panda et al. 2011). Therefore, special attention should be paid to experimental conditions when evaluating the effect of alcohol on autophagy.

Using an acute-binge ethanol gavage model (33% v/v, 4.5g/kg), we demonstrated that ethanol treatment increased autophagosome numbers by electron microscopy, assessment of GFP-LC3 positive autophagosomes by confocal microscopy, and detection of LC3-II protein levels by Western blot analysis *in vivo* and *in vitro* (Ding, Li et al. 2010). Intriguingly, we further demonstrated that acute ethanol-induced autophagy selectively removed damaged mitochondria and excess lipid droplets, but not long-lived proteins (Ding, Li et al. 2011). Induction of autophagy by ethanol is mediated by ethanol-induced production of ROS and inhibition of mTOR, and induction of autophagy requires ethanol metabolism.

Other recent studies also found that chronic ethanol treatment increased autophagosome content and autophagic flux in mouse livers and cultured hepatocytes (Lin, Zhang et al. 2013, Thomes, Ehlers et al. 2013). Otsuki's group demonstrated that autophagy was protective in rats fed the Lieber-DeCarli ethanol diet for 10 weeks. This group noticed an

induction of autophagosomes engulfing damaged mitochondria or lipids in addition to several lysosomes containing degraded organelles in ethanol-fed rats compared to control rats by electron microscopy. Interestingly, they found several autophagosomes that contained both mitochondria and lipid droplets, which suggest that these degradative pathways may be linked. They also saw an induction of LC3-II puncta and an increase in autophagosome-lysosome fusion after ethanol treatment compared to controls (Eid, Ito et al. 2013). However, this study lacked an autophagy flux assay, so whether the accumulation of autophagosomes was due to the induction of autophagy by ethanol treatment is not known. Lin *et al.* also showed that chronic ethanol consumption activates autophagy using mice fed the Lieber-DeCarli diet for 4 weeks. They showed that autophagy was activated in the chronic feeding model using an autophagy flux assay, where co-treatment with CQ and ethanol feeding increased GFP-LC3 puncta and protein levels more than ethanol feeding alone. However, they only treated mice with the ethanol diet for 4 weeks, so the role of ethanol feeding for a longer period of time on autophagy should be more critically evaluated (Lin, Zhang et al. 2013).

In contrast to the evidence supporting acute and chronic ethanol induction of autophagy, other studies suggest that ethanol may suppress hepatic autophagy (Donohue 2009, Fortunato, Burgers et al. 2009, Wu, Wang et al. 2012). It is well known that chronic alcohol consumption can cause hepatomegaly and protein accumulation (Baraona, Leo et al. 1975, Donohue 2009), which would suggest impaired autophagy. However, it should be noted that alcohol consumption inhibits hepatic proteasome activity, another important cellular catabolic pathway in addition to autophagy (Donohue, Zetterman et al. 1998, Dolganiuc, Thomes et al. 2012, Thomes, Trambly et al. 2012). Moreover, there is crosstalk between proteasome-mediated degradation and autophagy, and proteasome inhibition can increase autophagy as a compensatory mechanism (Ding, Ni et al. 2007, Ding and Yin 2008, Wu, Cho et al. 2010). Therefore, chronic alcohol consumption-induced accumulation of hepatic proteins and

hepatomegaly could be due to multiple factors and might not be due simply to impaired autophagy. While Cederbaum's group recently reported that acute ethanol inhibited autophagy, his findings were only based on the observations that ethanol treatment decreased LC3-II protein and LC3 positive puncta levels, and an autophagy flux assay was not implemented (Wu, Wang et al. 2012, Yang, Wu et al. 2012). Despite the controversy on the autophagy status of acute versus chronic alcohol exposure conditions, all studies unanimously demonstrated a beneficial role for autophagy in protecting against alcohol-induced steatosis and hepatotoxicity. Therefore, pharmacological induction of autophagy may be a promising approach for treating ALD.

In addition to hepatocytes, there are many other cell types such as hepatic stellate cells (HSCs) and macrophages in the liver that also play a role in the pathogenesis of ALD. Emerging evidence indicates that autophagy in these and other cell types in the liver may also be critical in liver physiology and pathogenesis. HSCs are one of the key cells responsible for regulating hepatic fibrosis, and recent evidence suggests that autophagy in HSCs promotes liver fibrosis by providing free fatty acids as an energy source for HSC activation through lipophagy (Hernandez-Gea, Ghiassi-Nejad et al. 2012). Cre-induced specific deletion of Atg7 in HSCs attenuated CCl_4-induced fibrosis *in vivo* (Hernandez-Gea, Ghiassi-Nejad et al. 2012). The decreased fibrogenic capacity of HSCs by inhibiting autophagy was also confirmed *in vitro* using primary cultured HSCs and immortalized HSC cell lines (Thoen, Guimaraes et al. 2011, Hernandez-Gea, Ghiassi-Nejad et al. 2012). Interestingly, a study from Friedman's group recently showed that autophagy was activated in HSCs in an 8-week chronic ethanol feeding model in rats. They showed that ER stress is induced in HSCs isolated from ethanol-fed rats, and that this ER stress further induced autophagy activation and subsequent HSC activation (Hernandez-Gea, Hilscher et al. 2013). These results imply that chronic ethanol-induced autophagy in HSCs may promote fibrosis during the pathogenesis of ALD. In contrast to HSCs,

specific deletion of autophagy in macrophages was also reported to exacerbate CCl_4-induced fibrosis in mouse livers by promoting HSC activation through enhanced secretion of inflammatory cytokines from macrophages (Mallat, Lodder et al. 2014). Moreover, we also found that hepatocyte-specific Atg5 knockout mice had severe liver injury, and these mice develop fibrosis (Ni, Woolbright et al. 2014). However, due to a lack of proper animal models to study fibrosis in ALD, it is not yet clear how modulating autophagy would affect fibrosis in ALD pathogenesis.

Chapter 2. Specific Aims

2.1. Specific Aim 1: Determine the Role of Parkin in Acetaminophen-induced Liver Injury

We have previously shown that autophagy protects against acetaminophen-induced liver injury (discussed in section 1.6.5) by removing damaged mitochondria via mitophagy. However, the mechanism for removal of these mitochondria is unknown. My preliminary data suggested that Parkin-induced mitophagy is important for protection against acetaminophen-induced liver injury because Parkin was recruited from the cytosol to the mitochondria after acetaminophen treatment. In addition, mitochondrial protein ubiquitination increased after acetaminophen treatment, indicating that Parkin was performing its E3 ligase function. Therefore, my goal for Aim 1 was to determine the role of Parkin-induced mitophagy in protection against acetaminophen-induced liver injury. The objective of this aim was to determine if Parkin is important for the mitophagy that occurs in acetaminophen-induced liver injury and if an alternative mechanism for mitophagy induction occurs in the absence of Parkin during acetaminophen-induced liver injury using WT and Parkin KO mice. *I hypothesized that Parkin-induced mitophagy is a protective mechanism in acetaminophen-induced liver injury.*

2.2. Specific Aim 1: Sub-Aim 1.

Surprisingly, my preliminary data showed that Parkin KO mice were protected against APAP-induced liver injury, and this protection was not due to differences in APAP metabolism between WT and Parkin KO mice. My goal was to determine the mechanisms by which Parkin KO mice are protected against acetaminophen-induced liver injury. As discussed in section 1.5.5, Parkin negatively regulates Cyclin D1 and Cyclin E (Veeriah, Morris et al. 2010, Yeo, Ng et al. 2012). In addition, Parkin expression is down-regulated in various cancers, including hepatocellular carcinoma (Wang, Denison et al. 2004, Fujiwara, Marusawa et al. 2008), and aged Parkin-deficient mice develop spontaneous liver tumors (Fujiwara, Marusawa et al. 2008). Therefore, the objective of this aim was to determine if Parkin regulates cellular proliferation in the acetaminophen-induced liver injury model. Differences in regulation of other pathways

involved in acetaminophen-induced liver injury, such as JNK activation, were also evaluated for WT and Parkin KO mice. *I hypothesized that Parkin KO mice are protected against acetaminophen-induced liver injury due to an upregulation of proliferative proteins in the absence of Parkin.* A scheme for Aim 1 and sub-Aim 1 is shown in **Figure 2.2.**

Figure 2.2

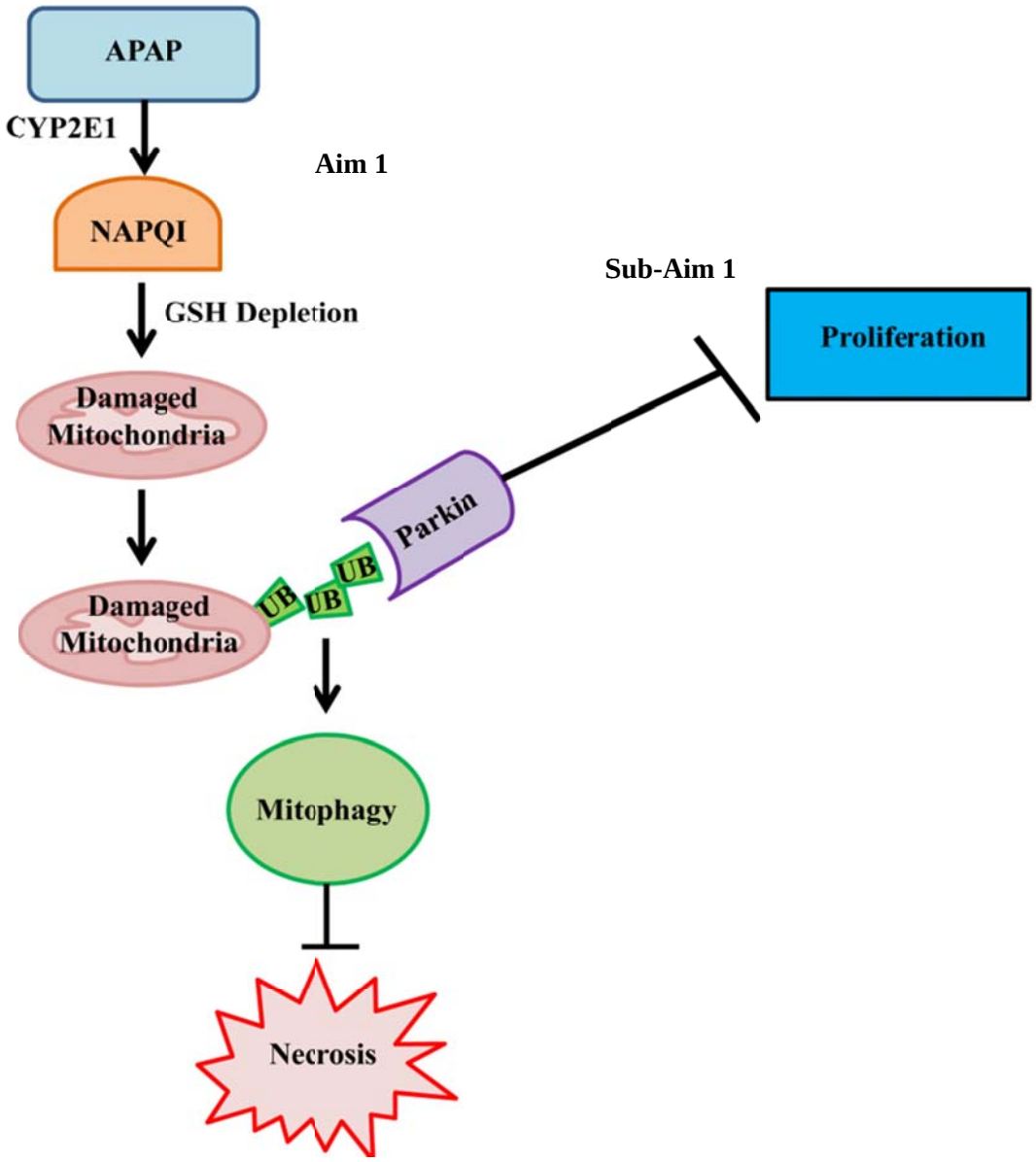

Figure 2.2: Scheme for Aim 1 and Sub-Aim 1

2.3. Specific Aim 2: Determine the Role of Parkin in Alcohol-induced Liver Injury

We and others have previously shown that autophagy protects against alcohol-induced liver injury by removing damaged mitochondria via mitophagy (discussed in section 1.7.3). However, the mechanism of mitophagy induction in the liver after alcohol treatment is unknown. My preliminary data showed that Parkin protected against ethanol-induced liver injury and steatosis in the acute-binge model, and Parkin's protection against ethanol-induced steatosis was not due to decreased lipid synthesis. We also found that Parkin translocated to mitochondria after ethanol treatment. Therefore, *I hypothesized that Parkin attenuates ethanol-induced liver injury and steatosis by promoting selective removal of damaged mitochondria (mitophagy), which allows for prevention of cell death and maintenance of mitochondrial function*. A scheme for Specific Aim 2 is shown in **Figure 2.3.**

Figure 2.3

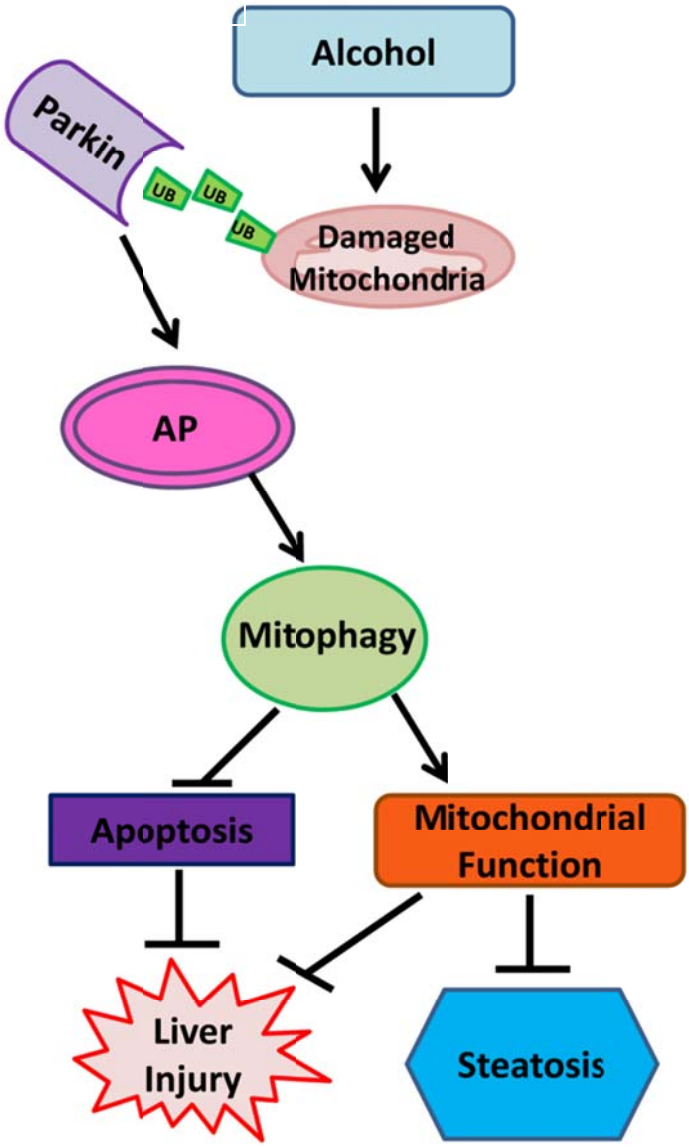

Figure 2.3: Scheme for Specific Aim 2

Chapter 3. Chronic Deletion and Acute Knockdown of Parkin have Differential Responses to Acetaminophen-induced Mitophagy and Liver Injury in Mice

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3.1 Abstract

We previously demonstrated that pharmacological induction of autophagy protected against acetaminophen (APAP)-induced liver injury in mice by clearing damaged mitochondria. However, the mechanism for this mitochondria removal by autophagy is unknown. Parkin, an E3 ubiquitin ligase, has been shown to be required for mitophagy induction in cultured mammalian cells following mitochondrial depolarization, but its role *in vivo* is not clear. The purpose of this study was to investigate the role of Parkin-mediated mitophagy in protection against APAP-induced liver injury. We found that Parkin translocated to mitochondria in mouse livers after APAP treatment followed by mitochondrial protein ubiquitination and mitophagy induction. To our surprise, we found that mitophagy still occurred in Parkin knockout (KO) mice after APAP treatment based on electron microscopy analysis and Western blot analysis for mitochondrial proteins, and Parkin KO mice were protected against APAP-induced liver injury compared to wild type mice. Mechanistically, we found that Parkin KO mice had decreased activated c-Jun N-terminal kinase (JNK), increased induction of myeloid leukemia cell differentiation protein (Mcl-1) expression, and increased hepatocyte proliferation after APAP treatment in their livers compared to WT mice. In contrast to chronic deletion of Parkin, acute knockdown of Parkin in mouse livers using adenovirus-shRNA reduced mitophagy and Mcl-1 expression but increased JNK activation after APAP administration, which exacerbated APAP-induced liver injury. Therefore, chronic deletion (KO) and acute knockdown of Parkin differentially regulate APAP-induced mitophagy and liver injury in mice.

3.2 Introduction

APAP overdose is the main cause of acute liver failure in the United States and can even lead to death (Larson 2007). APAP is metabolized by cytochrome P450s (Cyp), mainly by CYP2E1, to the reactive metabolite NAPQI (*N*-acetyl-*p*-benzoquinone imine), which is bound and detoxified by glutathione (GSH) after therapeutic doses of APAP. APAP overdose causes GSH depletion and allows for NAPQI to bind to proteins, which leads to mitochondrial dysfunction and hepatocyte necrosis (McGill, Sharpe et al. 2012). APAP was extensively discussed in section 1.6. We previously found that pharmacological induction of autophagy via rapamycin was protective against APAP-induced liver injury, likely by removing damaged mitochondria (Ni, Boggess et al. 2012). However, the mechanism for removal of these damaged mitochondria in the liver is unknown.

As previously discussed in section 1.2, autophagy is an evolutionarily conserved process that results in degradation of cellular proteins and organelles due to a cell's "self-eating". In addition to providing the cell with nutrients and energy in response to starvation, this process rids the cell of misfolded proteins and damaged organelles through the formation of double-membrane autophagosomes. Autophagosomes can engulf individual organelles, protein aggregates, or portions of cytoplasm before fusing with lysosomes to degrade their contents (Parzych and Klionsky 2014). Autophagy is a protective process that can be either selective or non-selective. Non-selective autophagy occurs during starvation to break down the cell's components in order to provide a source of energy and nutrients. Selective autophagy occurs in nutrient-rich or poor conditions as a protective mechanism by ridding the cell of protein aggregates and damaged organelles (Reggiori, Komatsu et al. 2012). Mitophagy is a selective form of autophagy that is specific for removal of damaged mitochondria, and mitophagy has been shown *in vitro* to be mediated by the E3 ubiquitin ligase Parkin. Parkin is recruited to damaged mitochondria by PTEN-induced putative kinase 1 (PINK1) to initiate their removal by

mitophagy by performing K48 and K63 ubiquitination of mitochondrial outer membrane proteins (Narendra, Tanaka et al. 2008, Geisler, Holmstrom et al. 2010, Matsuda, Sato et al. 2010, Narendra, Jin et al. 2010, Vives-Bauza, Zhou et al. 2010, Chan, Salazar et al. 2011) (discussed in section 1.3.2).

Parkin-induced mitophagy is mainly known for its protective role in the brain because loss of Parkin has been linked to Autosomal Recessive Parkinsonism (Kitada, Asakawa et al. 1998). We recently found that parkin is also ubiquitously expressed in several tissues in mouse, including the liver (Ding and Yin 2012). Therefore, we investigated the role of Parkin in mitophagy induction as a mechanism of protection in APAP-induced liver injury. We found that Parkin-induced mitophagy is likely a mechanism of protection in APAP-induced liver injury because Parkin translocated to mitochondria and increased the level of mitochondrial protein ubiquitination after APAP treatment. However, we surprisingly found that Parkin knockout (KO) mice also had mitophagy in their livers after APAP treatment likely due to other compensatory mechanisms. In addition, Parkin KO mice were protected against APAP-induced liver injury compared to wild type (WT) mice. Mechanistically, we found that Parkin KO mice had decreased activation of JNK, increased induction of Mcl-1 expression, and increased proliferation, which are all known important factors in mediating APAP-induced necrosis and liver injury. In contrast to chronic deletion of Parkin, acute knockdown of Parkin in mouse livers resulted in reduced mitophagy and Mcl-1 expression but increased JNK activation after APAP administration, which exacerbated APAP-induced liver injury. Our results thus revealed that chronic deletion (KO) and acute knockdown of Parkin differentially regulate APAP-induced mitophagy and liver injury in mice.

3.3 Experimental Procedures

Materials. APAP was purchased from Sigma (A7085), and the kit for alanine aminotransferase (ALT) measurement was purchased from Pointe Scientific (A7526-450). The following antibodies were used for western blot analysis: anti-Parkin (Santa-Cruz, SC-32282), anti-Ubiquitin (Santa Cruz, SC-8017), anti-β-Actin (Sigma, A5441), anti-Cyp2e1 (Abcam, ab19140), anti-CyclinD1 (Lab Vision, RB-9041), anti-phosphorylated JNK (Cell Signaling, 4668S), anti-phosphorylated GSK-3β (Ser9) (Cell Signaling, 5558), anti-GSK-3α/β (Cell Signaling, 5696), anti-phosphorylated glycogen synthase (Cell Signaling, 4858), anti-JNK (BD, 554285), anti-CoxIV (Mitosciences, MS407), anti-Mcl-1 (Rockland, 600-401-394), anti-Tom20 (Santa-Cruz, SC11415), anti-Cyclophilin D (Mitosciences, E0667), and anti-GAPDH (Cell Signaling, 2118). The APAP-adduct antibody was a gift from Dr. Lance Pohl (NIH) (Matthews, Roberts et al. 1996, Ryan, Bourdi et al. 2012). Anti-PCNA antibody (Santa-Cruz, SC-56) was used for immunostaining. Horseradish peroxidase or biotin-conjugated antibodies were from Jackson ImmunoResearch Lab. Adenovirus (Ad) negative shRNA and Ad shRNA for mouse Parkin were purchased from Vector Biolabs (Malvern, PA).

Animal Experiments. WT C57BL/6J (#000664) and whole body Parkin KO mice (C57BL/6J background, #006582) were purchased from the Jackson Laboratory. All animals received humane treatment, and all protocols were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Eight to 12 week old male mice were treated with either 500 mg/kg of APAP or saline by IP injection and were sacrificed 0.5, 1, 2, 6, or 24 hours after treatment. To achieve knockdown of Parkin in mouse livers, two month old male C57BL/6J mice were injected intravenously via tail vein with Ad-negative (Neg) shRNA or Ad-Parkin shRNA (1×10^9 PFU per mouse) for 4 days. Then the mice were further treated with either APAP (500 mg/kg) or saline by IP injection for 6 hours. Liver injury was determined by

measuring serum ALT. Formalin-fixed liver sections were embedded in paraffin and cut into 5 μm sections before staining with hematoxylin and eosin (H&E) to determine liver cellular necrosis.

Western Blot Analysis. Total liver lysates were prepared using RIPA buffer. Heavy membrane (HM) mainly enriched with mitochondria and cytosolic fractions were prepared as described previously (Ding, Ni et al. 2004). Briefly, liver tissues were homogenized in HIM buffer (200 mM mannitol, 70 mM sucrose, 5 mM Hepes, 0.5 mM EGTA (pH 7.5) containing protease inhibitors) using a dounce homogenizer. Homogenates were centrifuged at 1,000 x g to remove debris, and the supernatant was centrifuged at 10,000 x g for 10 minutes to separate HM and cytosolic fractions. The supernatant was kept as the cytosolic fraction, and the pellet containing the HM fraction was further washed by centrifugation and re-suspended in HIM buffer. Protein (20-30 μg) was separated by a 12% SDS-PAGE gel before transfer to a PVDF membrane. Membranes were probed using indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

Proliferating Cell Nuclear Antigen (PCNA) Staining. Formalin-fixed liver sections were embedded in paraffin and cut into 5 μM sections before staining with anti-PCNA antibody. Sections were deparaffinized in xylene and dehydrated in ethanol followed by incubation in hydrogen peroxide to quench peroxidases. Tissues were then blocked using Ultra V-Block (Thermo Scientific, TA-125-UB) for 5 minutes and incubated with PCNA antibody (1:100) overnight at 4°C. Sections were further incubated with biotinylated secondary antibody for 30 minutes, and PCNA-positive cells were detected using ABC reagent (Vector, PK-6100) and DAB peroxidase substrate (Vector, SK-4105). Sections were counterstained with hematoxylin

(Sigma, GHS132) for 30 seconds. Six fields at 200 x magnification were quantified for PCNA positive cells per tissue, and results were expressed as percent PCNA positive hepatocytes.

Culture of Primary Hepatocytes. As described previously (Ni, Bockus et al. 2012), murine hepatocytes were isolated from WT and Parkin KO mice by a retrograde, non-recirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma). Cells were cultured in William's medium E with 10% fetal bovine serum but no other supplements for 2 hrs for attachment. Cells were then cultured in the same medium without serum overnight before treatment. All cells were maintained in a 37°C incubator with 5% CO_2. For assessing mitochondrial membrane potential, hepatocytes were first loaded with Tetramethylrhodamine, methyl ester (*TMRM*) (50 nM) for 15 minutes and then treated with APAP (0, 5, 10 mM) for 8 hours. Cells were further stained with Hoechst 33342 (1 μg/mL) for 5 minutes followed by fluorescence microscopy. For assessing necrosis after APAP treatment, hepatocytes were stained with propidium iodide (PI, 1 μg/mL) for 5 minutes followed by fluorescence microscopy.

Caspase-3 Activity. Caspase-3 activity was determined using Ac-DEVD-AFC fluorescent substrate (Enzo) and 20 μg of protein from mouse liver lysates after treatment with saline or APAP as we previously described (Ding, Ni et al. 2004). Liver lysate from an Atg5 KO mouse was used as a positive control (Ni, Boggess et al. 2012).

GSH Measurement. Glutathione (GSH) and glutathione disulfide (GSSG) levels in liver tissue were measured using a modified Tietze assay (Jaeschke and Mitchell 1990). For GSH measurement, liver tissues were homogenized in sulfosalicylic acid (3%) followed by centrifugation and dilution in potassium phosphate buffer. Samples were then subjected to a cycling reaction using glutathione reductase and dithionitrobenzoic acid, and GSH levels were

determined by spectrophotometry. For GSSG, reduced GSH was removed using N-ethylmaleimide, and the GSSG measurement was performed using a similar spectrophotometer method as for GSH.

Analysis of Proteasome Activity. Proteasome activity was determined using a Suc-LLVY-AMC substrate (Enzo) for the 20s proteasome subunit and 20 μg of protein from mouse liver lysates after treatment with APAP or saline as previously described (Williams, Hou et al. 2013).

Electron microscopy. Tissues were fixed with 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) followed by 1% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1016CX electron microscope.

Statistical Analysis. Statistical analysis was conducted with Student's *t* test or one-way ANOVA analysis where appropriate. A $p<0.05$ was considered significant.

3.4 Results

3.4.1 Parkin Translocated to Mitochondria after APAP Treatment, but Parkin KO Mice were Resistant to APAP-induced Liver Injury.

During Parkin-mediated mitophagy in cultured cells, Parkin translocates from the cytosol to depolarized mitochondria resulting in mitochondrial degradation (Narendra, Tanaka et al. 2009, Ding, Ni et al. 2010, Vives-Bauza, Zhou et al. 2010). We previously showed that APAP administration induces mitophagy in mouse livers (Ni, Bockus et al. 2012). Therefore, we investigated the role of Parkin in mitophagy induction in the liver after APAP treatment. We first treated WT mice with APAP (500 mg/kg) for 6 hours and determined Parkin translocation to mitochondria by western blot analysis using cytosolic and mitochondrial fractions. Parkin translocated to mitochondria after APAP treatment, which was accompanied by increased ubiquitination of mitochondrial proteins, suggesting that Parkin may play a role in APAP-induced mitophagy (**Figure 3.4.1 A**).

To determine if Parkin-induced mitophagy was a protective mechanism against APAP-induced liver injury, we treated WT and Parkin KO mice with APAP (500 mg/kg) for 6 and 24 hours. We expected Parkin KO mice to have increased liver injury after APAP treatment due to an inability to induce mitophagy. However, we surprisingly found that Parkin KO mice were protected from APAP-induced liver injury compared to WT mice (**Figure 3.4.1 B-C**). Parkin KO mice had significantly less liver injury compared to WT mice after APAP treatment for 6 and 24 hours as determined by serum ALT levels. WT mice had a mean serum ALT of 5309 U/L at 6 hours and 8290 U/L at 24 hours, and KO mice had a mean serum ALT of 2813 U/L at 6 hours and 2649 U/L at 24 hours (**Figure 3.4.1 B**). WT mice also had a significantly greater area of hepatocellular necrosis than KO mice after APAP treatment for 6 and 24 hours as determined by H&E staining. Saline treated WT and Parkin KO mice did not develop any cellular necrosis (**Figure 3.4.1 C**).

APAP is well known to induce cell death via necrosis and not apoptosis (McGill and Jaeschke 2013), so we ensured that protection in Parkin KO mice was not due to APAP induction of cell death via apoptosis instead of necrosis by assessing caspase-3 activity in liver tissue lysates. Neither WT nor Parkin KO mice had any induction of caspase-3 activity after APAP treatment, indicating that APAP did not induce apoptosis in WT or Parkin KO mice, as expected. Lysate from an Atg5 KO mouse was used as a positive control for caspase-3 activation (**Figure 3.4.1 D**).

Figure 3.4.1

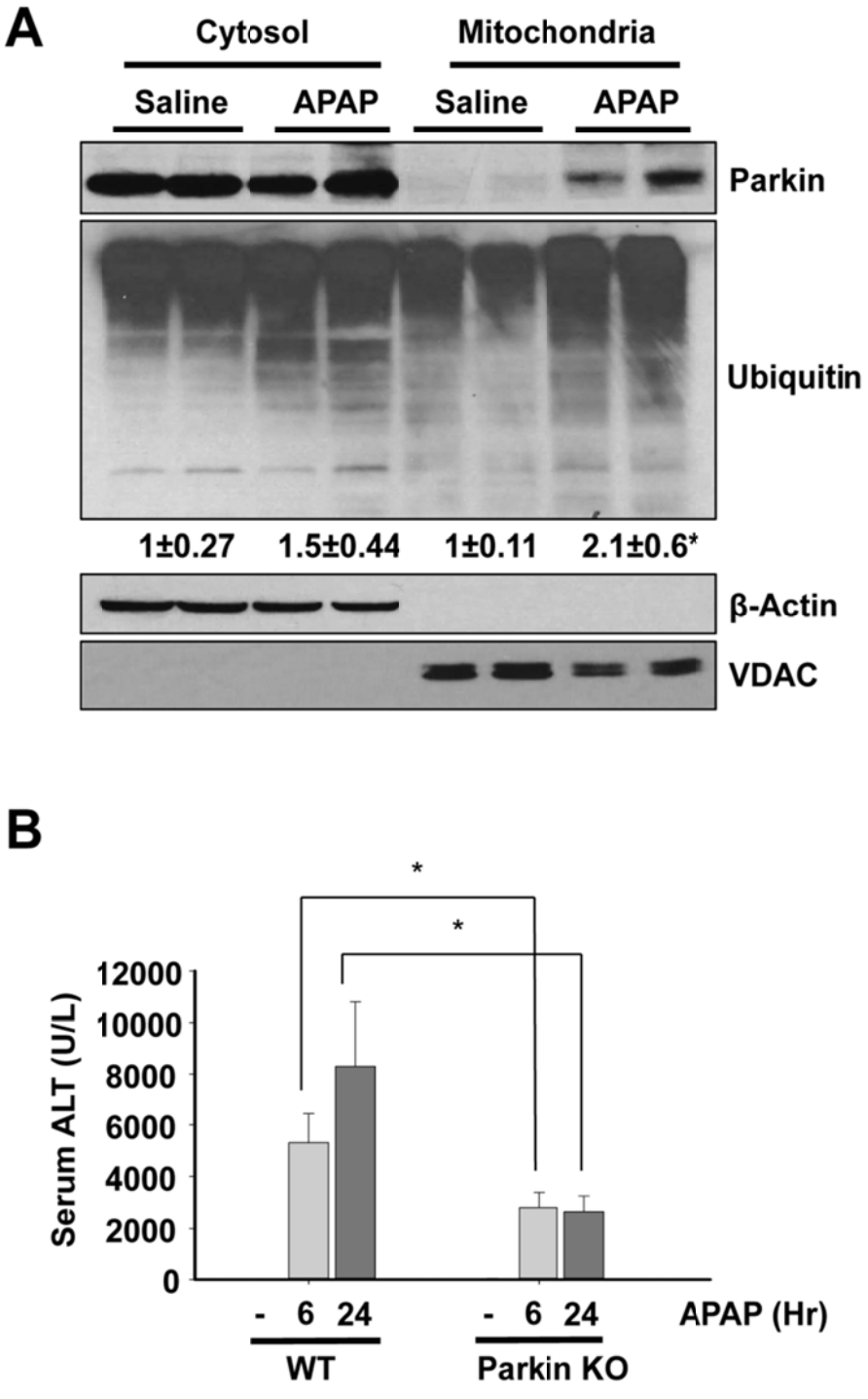

A
Cytosol
Mitochondria
Saline
APAP
Saline
APAP
Parkin
Ubiquitin
1±0.27
1.5±0.44
1±0.11
2.1±0.6*
β-Actin
VDAC
B
*
*
Serum ALT (U/L)
12000
10000
8000
6000
4000
2000
0
- 6 24
- 6 24
APAP (Hr)
WT
Parkin KO

Figure 3.4.1 Cont.

C

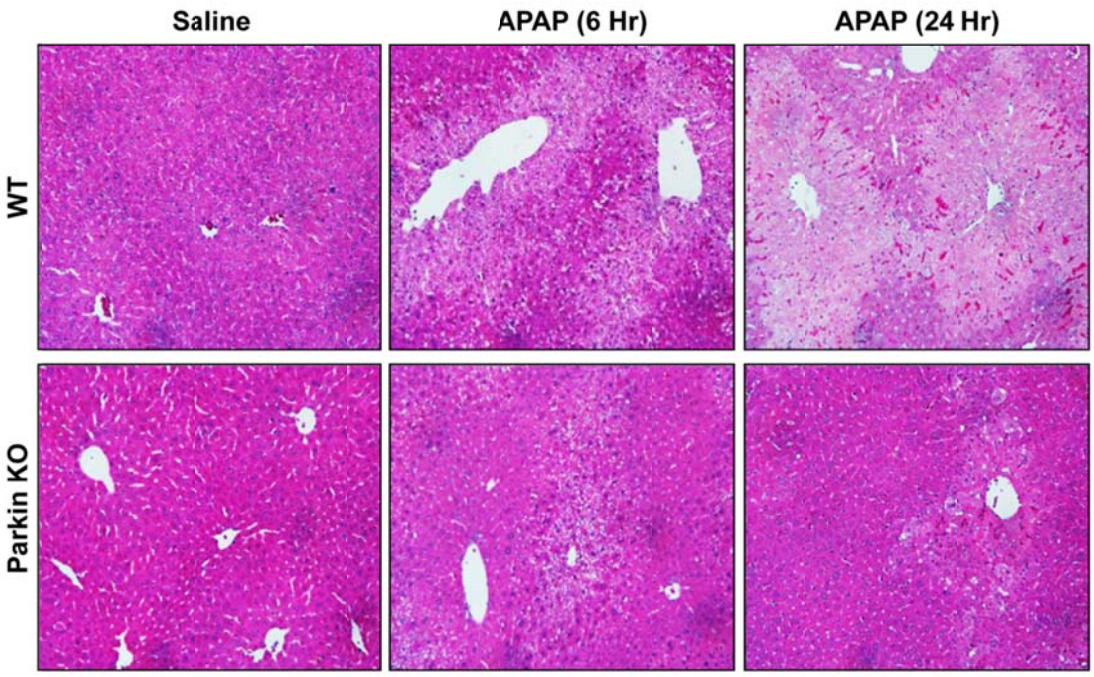


Figure 3.4.1 Cont.

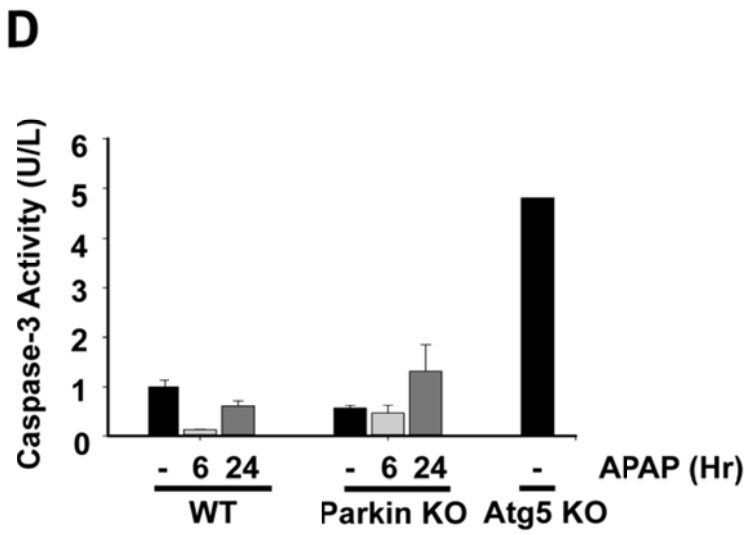


Figure 3.4.1: Parkin KO mice were resistant to APAP-induced liver injury. A) WT mice were treated with 500 mg/kg APAP or saline control for 6 hours and liver cytosolic and mitochondrial fractions were isolated and analyzed by Western blot. Data from two representative mice are shown. β-actin and VDAC were used as loading controls for cytosolic and mitochondrial fractions, respectively. Densitometry quantification is for ubiquitin Western blots. Data shown are Means ± SE (n=5 for each group, * =p<0.05). Results were normalized to Gapdh. **B)** WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 or 24 hours and blood samples were measured for serum ALT. Data shown are Means ± SE (n $\geq$ 5, * =p<0.05). **C)** Representative H&E images are shown from WT and Parkin KO mice treated with 500 mg/kg APAP or saline control for 6 or 24 hours (100x magnification). **D)** WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 and 24 hours, and caspase-3 activity was measured using liver lysate. Liver lysate from a saline treated Atg5 KO mouse was used as a positive control. Data shown are Means ± SE (n=3, no significant differences between groups).

3.4.2 Protection in Parkin KO Mice was not due to Differences in APAP Metabolism or Oxidative Stress.

APAP is metabolized primarily by CYP2E1 to its reactive metabolite NAPQI, which is detoxified by GSH at therapeutic doses. GSH is depleted during APAP overdose, which allows for NAPQI to form protein adducts and induce mitochondrial damage and subsequent hepatocellular necrosis (McGill and Jaeschke 2013). To ensure that protection in KO mice was not due to differences in APAP metabolism, we treated WT and Parkin KO mice with APAP (500 mg/kg) or saline control and measured GSH levels 0.5, 1, 2, 6, and 24 hours after treatment. WT and Parkin KO mice had similar basal levels of GSH and similar GSH depletion at 0.5, 1, and 2 hours after APAP treatment. WT and Parkin KO mice also had similar recovery of GSH after APAP treatment for 6 and 24 hours (**Figure 3.4.2 A**). Furthermore, WT and Parkin KO mice had similar levels of protein adducts and Cyp2e1 protein expression after APAP or saline treatment for 6 hours (**Figure 3.4.2 B**). These results suggest that protection in Parkin KO mice was not due to differences in metabolism of APAP between WT and Parkin KO mice.

Glutathione disulfide (GSSG) levels and the ratio of GSSG to GSH are markers of oxidative stress. WT and Parkin KO mice had similar basal GSSG levels and GSSG to GSH ratio. WT and Parkin KO mice also had similar GSSG levels and GSSG to GSH ratios after APAP treatment for 0.5, 1, 2, and 6 hours. Parkin KO mice had slightly less GSSG than WT mice after APAP treatment for 24 hours, but the decrease in KO mice was not significant compared to WT mice (**Figure 3.4.2 C-D**). These results suggest that differences in injury between WT and Parkin KO mice were not due to differences in oxidative stress.

Figure 3.4.2.

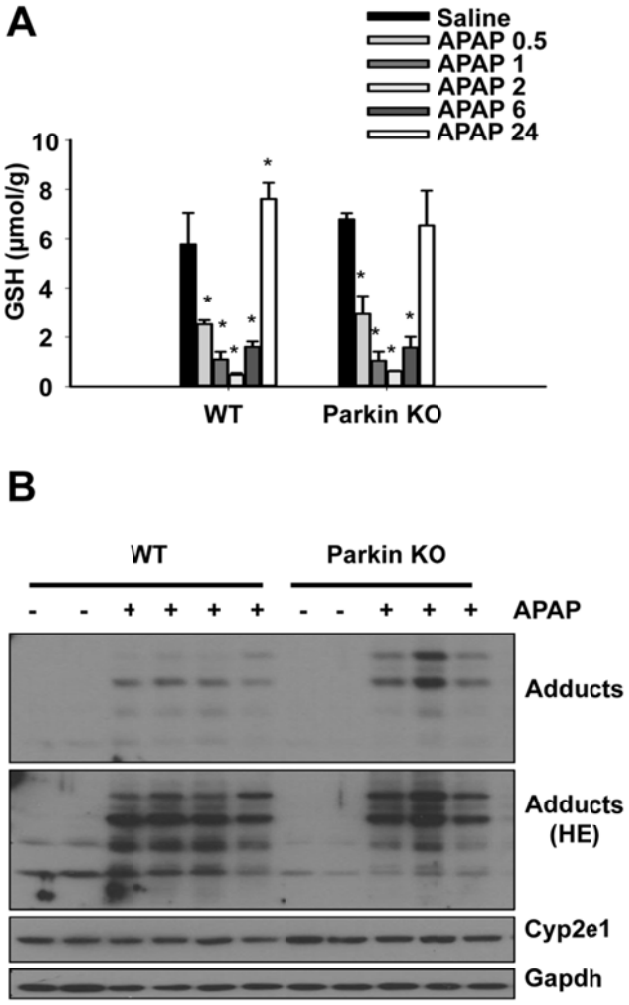

A
Saline
APAP 0.5
APAP 1
APAP 2
APAP 6
APAP 24
GSH (µmol/g)
10
8
6
4
2
0
WT
Parkin KO
B
WT
Parkin KO
- - + + + + - - + + + APAP
Adducts
Adducts (HE)
Cyp2e1
Gapdh

Figure 3.4.2. Cont.

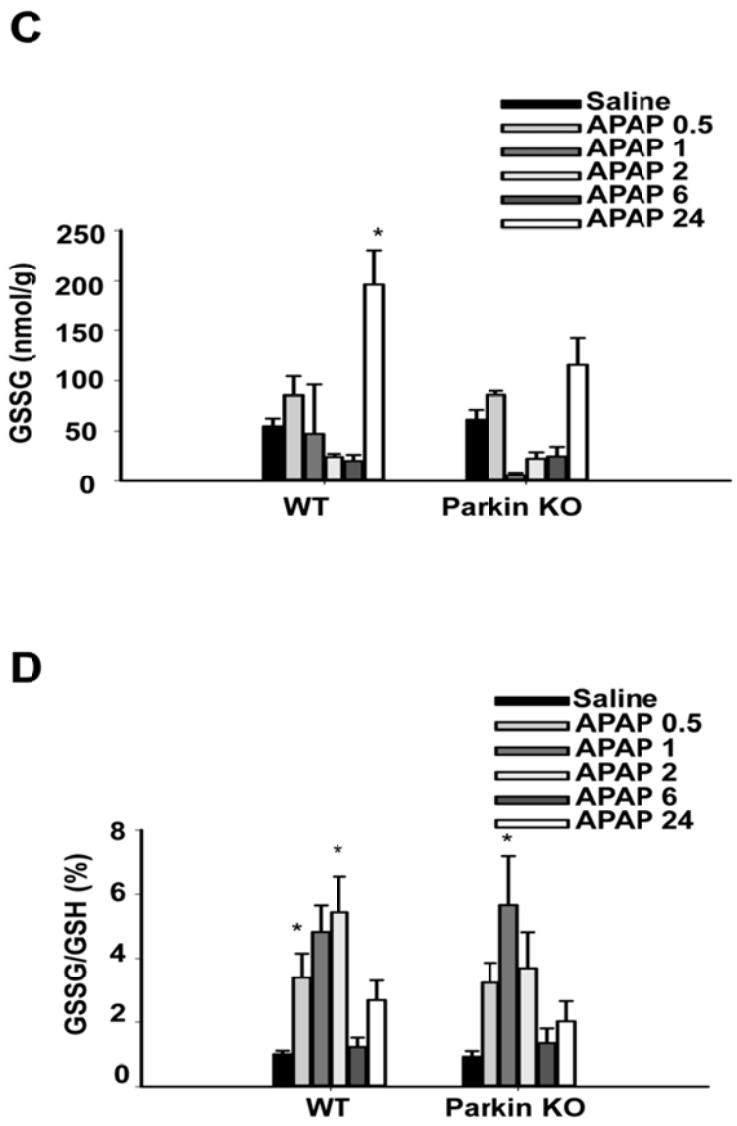


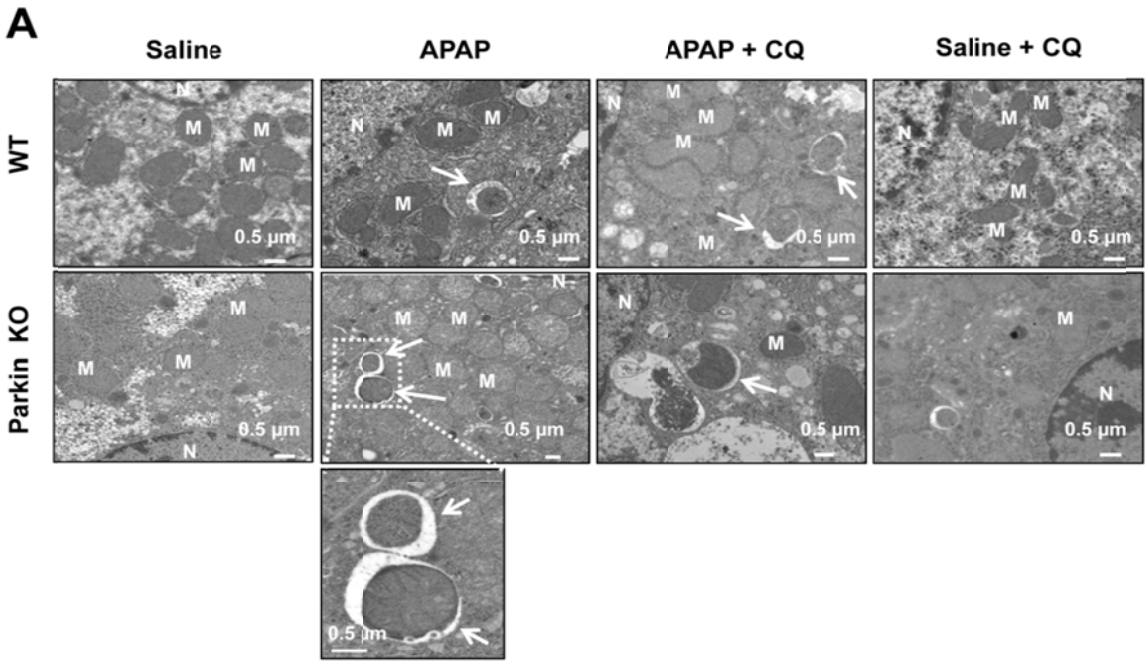
Figure 3.4.2: Protection in Parkin KO mice was not due to differences in APAP metabolism or oxidative stress. A) WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 0.5, 1, 2, 6, or 24 hours before measurement of liver GSH. Data shown are Means ± SE ($n \geq 3$, *=$p<0.05$ compared to individual saline controls, no significant differences between WT and Parkin KO mice). **B)** WT and Parkin KO mice were treated with 500 mg/kg APAP for 6 hours, and total liver lysates from individual mice were analyzed by Western blot. Gapdh was used as a loading control (HE=high exposure). **C)** WT and Parkin KO mice were treated as in "A", and liver GSSG was measured. Data shown are Means ± SE ($n \geq 3$, *=$p<0.05$ compared to WT saline control, no significant differences between WT and Parkin KO mice). **D)** The ratio from results for GSH in "A" and GSSG in "C" were further calculated. Data shown are Means ± SE (*=$p<0.05$ compared to individual saline controls, no significant differences between WT and Parkin KO mice).

3.4.3 Mitophagy Occurred in Both WT and Parkin KO Mice after APAP Treatment

We compared and quantified the number of autophagosomes and lysosomes containing mitochondria (hereafter referred to as mitophagosomes) by electron microscopy (EM) between WT and Parkin KO mice after APAP treatment. We also performed an autophagy flux assay with a combination of APAP and chloroquine (CQ) treatment. CQ blocks lysosome degradation, which allows for accurate quantification of autophagosome and lysosome components (Klionsky, Abdalla et al. 2012). Treatment with APAP significantly increased the number of mitophagosomes, which was further enhanced in the presence of CQ in WT mice, indicating that APAP induces mitophagy flux in mouse livers. Surprisingly, Parkin KO mice also had an increased number of mitophagosomes, which was also further enhanced by the presence of CQ treatment, although the number of mitophagosomes was lower compared to WT mice (**Figure 3.4.3 A-B**).

WT and Parkin KO mice also both showed slight degradation of the mitochondrial outer membrane protein Tom20 as well as the mitochondrial matrix protein Cyclophilin D after APAP treatment (**Figure 3.4.3 C-E**). We recently reported that APAP treatment in mice causes distinct mitochondrial changes in different zones of the mouse liver. In addition to mitophagy, APAP also induces mitochondrial biogenesis adjacent to portal areas likely to promote liver repair (Ni, Williams et al. 2013). This could offset the levels of mitochondrial degradation mediated by mitophagy using total liver lysates. A better quantitative approach, such as the use of a micro-dissection approach to isolate tissues from different zones of the liver, may need to be established in the future. Taken together, these results indicate that APAP may induce mitophagy in mouse livers through Parkin-dependent and-independent mechanisms.

Figure 3.4.3


A
Saline
APAP
APAP + CQ
Saline + CQ
WT
Parkin KO
N
M
0.5 μm

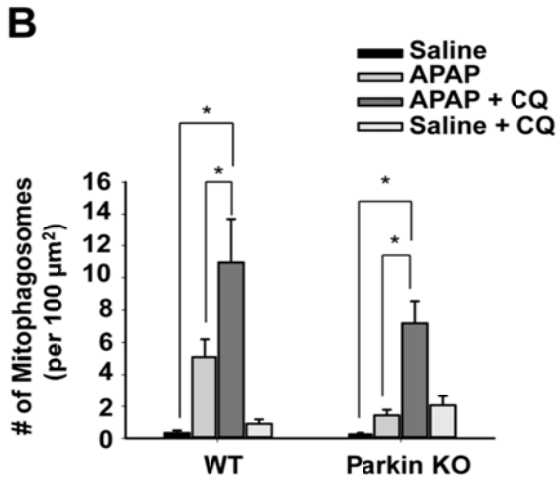

B
Saline
APAP
APAP + CQ
Saline + CQ
# of Mitophagosomes (per 100 μm²)
0
2
4
6
8
10
12
14
16
*
WT
Parkin KO

Figure 3.4.3 Cont.

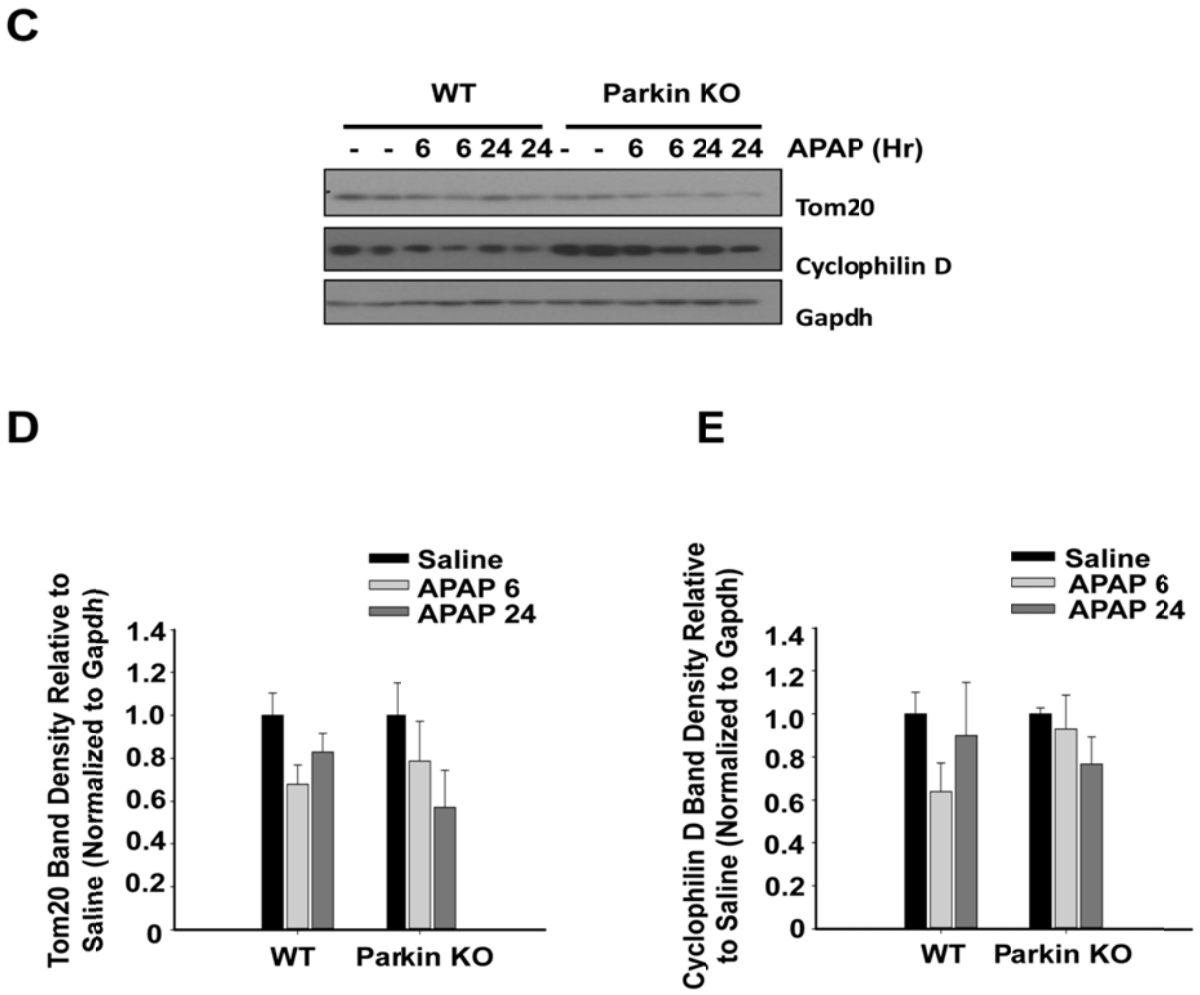


Figure 3.4.3: Mitophagy occurred in both WT and Parkin KO mice after APAP treatment. A) Representative EM images are shown from WT and Parkin KO mice treated with APAP (500 mg/kg) with or without CQ (60 mg/kg) for 6 hours. An enlarged image of mitophagosomes is shown from the dotted line boxed area (arrows=mitophagosomes, M=mitochondria, N=nucleus). **B)** Quantification of EM images. Data shown are Means ± SE ($n \geq 2$ mice per group, at least 10 images quantified per group, *=$p<0.05$, no significant differences between WT and Parkin KO mice). **C)** WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 and 24 hours, and total liver lysates were analyzed for mitochondrial protein degradation by Western blot. Gapdh was used as a loading control. Results for 2 individual mice are shown. **D-E)** Densitometry quantification of Western blots for Tom20 and Cyclophilin D. Data shown are Means ± SE (n=4 for each group, no significant differences among groups). Results were normalized to Gapdh.

3.4.4 Parkin KO Mice had Decreased JNK Activation Compared to WT Mice after APAP Treatment

Activation of JNK by phosphorylation and its translocation to the mitochondria are well known to exacerbate APAP-induced liver injury (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007, Hanawa, Shinohara et al. 2008, Saito, Lemasters et al. 2010). To determine if WT and Parkin KO mice had differences in JNK activation, we measured protein levels of phosphorylated JNK (pJNK) in WT and Parkin KO mouse livers after APAP treatment for 2 and 6 hours. Parkin KO mice had slightly less JNK activation 2 hours after APAP treatment compared to WT mice, and JNK activation was significantly less in Parkin KO mice compared to WT mice 6 hours after APAP treatment (**Figure 3.4.4 A-B**). WT and Parkin KO mice had similar levels of total JNK protein expression (**Figure 3.4.4 A**). In addition, we investigated mitochondrial JNK activation using cytosolic and mitochondrial fractions from WT and Parkin KO mouse liver lysates after APAP treatment for 6 hours. We found that JNK translocated to mitochondria after APAP treatment, and the level of total JNK levels on mitochondria was surprisingly higher in Parkin KO mice compared to WT mice (**Figure 3.4.4 C-D**). However, the active form of JNK (pJNK) on mitochondria was much lower in Parkin KO mice compared to WT mice after APAP treatment (**Figure 3.4.4 C-D**). These results suggest that Parkin may promote JNK activation once it translocates to mitochondria, and the regulation of JNK mitochondrial translocation is independent of Parkin.

Figure 3.4.4

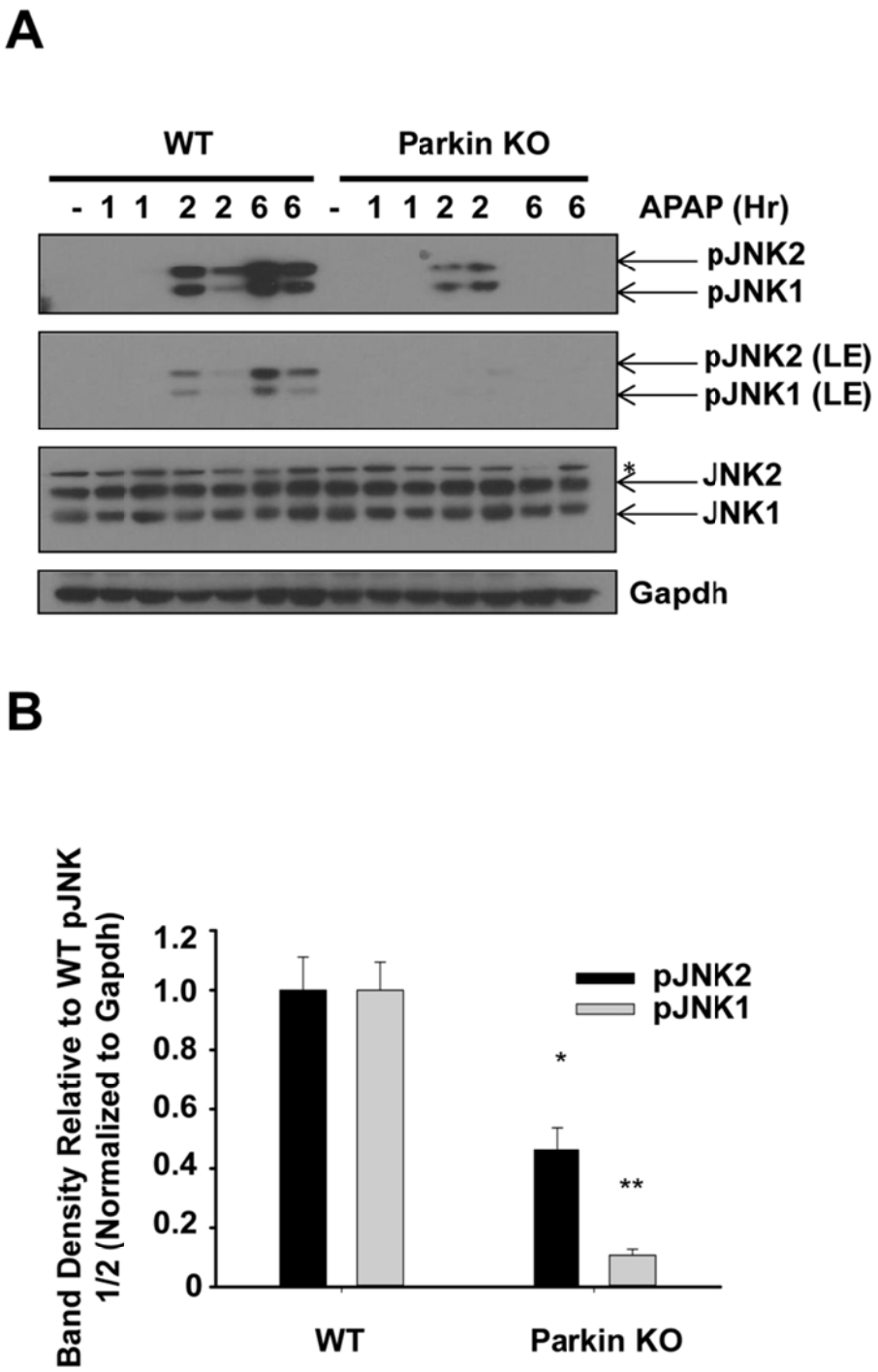

A
WT
Parkin KO
- 1 1 2 2 6 6 - 1 1 2 2 6 6
APAP (Hr)
pJNK2
pJNK1
pJNK2 (LE)
pJNK1 (LE)
*
JNK2
JNK1
Gapdh
B
Band Density Relative to WT pJNK 1/2 (Normalized to Gapdh)
1.2
1.0
0.8
0.6
0.4
0.2
0
pJNK2
pJNK1
*
**
WT
Parkin KO

Figure 3.4.4 Cont.

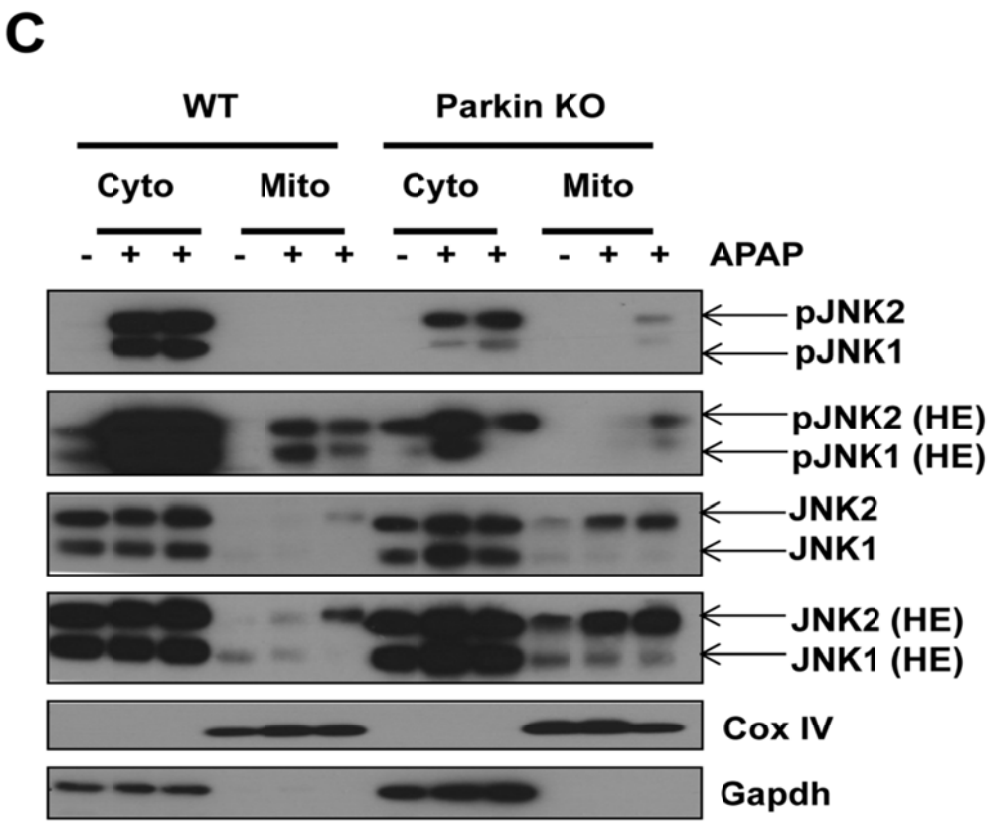


Figure 3.4.4 Cont.

D

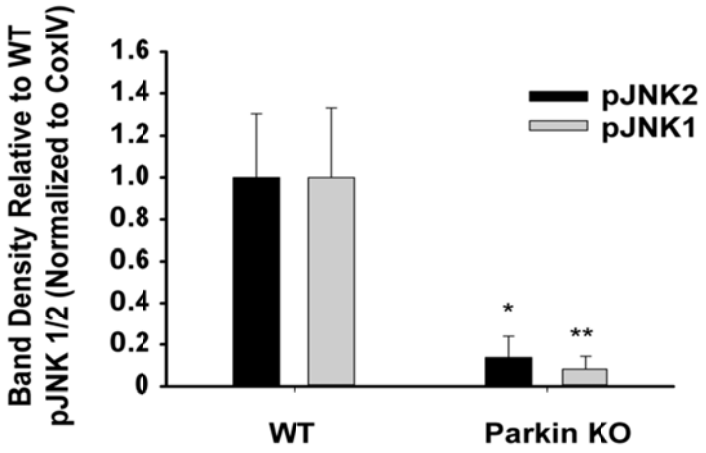


Figure 3.4.4: Protection in Parkin KO mice may be due to decreased JNK activation. A) WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 1, 2, or 6 hours, and total liver lysates were analyzed by Western blot. Results from 1 representative mouse are shown for saline, and results from 2 representative mice are shown for APAP treatment. Gapdh was used as a loading control (LE=low exposure). **B)** Densitometry quantification of pJNK. Data shown are Means ± SE (n= 2 each for WT and Parkin KO saline, 3 each for WT and Parkin KO APAP treated mice, *=p<0.05 compared to WT APAP treatment at 6 hours, **=P<0.05 compared to WT APAP treatment at 24 hours). Results were normalized to Gapdh. **C)** WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 hours, and cytosolic and mitochondrial fractions were analyzed by Western blot. CoxIV and Gapdh were used as loading controls for HM and cytosolic fractions, respectively (HE=high exposure). **D)** Densitometry quantification of pJNK in the mitochondrial fraction. Data shown are Means ± SE (n= 2 each for WT and Parkin KO saline treated mice, 3 each for WT and Parkin KO APAP treated mice, *=p<0.05 compared to WT APAP treatment at 6 hours, **=P<0.05 compared to WT APAP treatment at 24 hours). Results were normalized to CoxIV.

3.4.5 Parkin KO Mice had Increased Mcl-1 Expression Compared to WT Mice after APAP Treatment

Mcl-1 is an anti-apoptotic protein that has been shown to be protective against APAP-induced liver injury (Shinohara, Ybanez et al. 2010, Sharma, Gadang et al. 2012). We measured protein levels of Mcl-1 using total lysate from WT and Parkin KO mouse livers 6 hours after APAP treatment. Mcl-1 protein expression was increased after APAP treatment in both WT and Parkin KO mice, and Parkin KO mice had higher basal levels of Mcl-1, which were sustained after APAP treatment compared to WT mice (**Figure 3.4.5 A-B**). It has been reported that GSK-3β phosphorylates Mcl-1 and promotes its proteasomal degradation (Maurer, Charvet et al. 2006). We found that APAP treatment increased the phosphorylation levels of Akt and GSK-3β at serine 9 (Ser 9) in both WT and Parkin KO mouse livers. Since Akt-mediated Ser 9 phosphorylation of GSK-3β negatively regulates its activity, we also determined the phosphorylated levels of glycogen synthase (GS), which is one of the substrates of GSK-3β. We found that there was a dramatic reduction in phosphorylated levels of GS in Parkin KO but not WT mouse livers after APAP treatment (**Figure 3.4.5 A**), suggesting decreased GSK-3β activity in APAP-treated Parkin KO mouse livers.

It is known that Mcl-1 is degraded by the ubiquitin proteasome system (Nijhawan, Fang et al. 2003, Maurer, Charvet et al. 2006), and APAP treatment tended to slightly decrease proteasome activity in WT mice, but these changes did not reach statistical differences (**Figure 3.4.5 C**). In addition, Parkin KO mice overall tended to have slightly decreased proteasome activity although APAP treatment did not further alter the proteasome activity, and these changes were also not statistically different (**Figure 3.4.5 C**). Collectively, these data suggest that protection in Parkin KO mice against APAP-induced liver injury was likely due to decreased JNK activation and increased Mcl-1 expression.

Figure 3.4.5

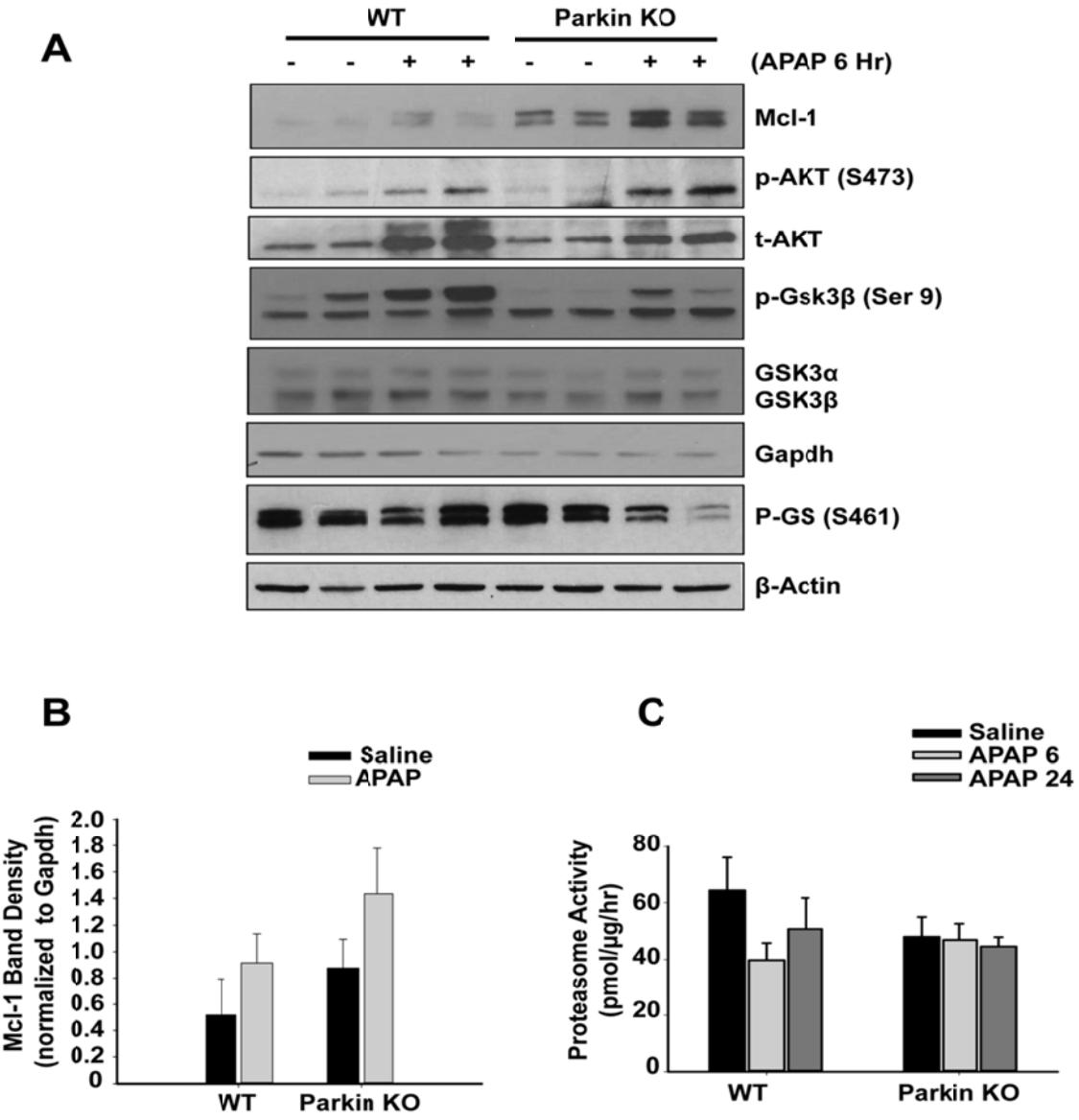


Figure 3.4.5: Parkin KO mice had increased levels of Mcl-1 proteins after APAP treatment. A) WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 hours, and total liver lysates were analyzed by Western blot. Gadph was used as a loading control. **B)** Densitometry quantification of Western blots for Mcl-1. Data shown are Means ± SE (n=4 per group). Results were normalized to Gapdh. C). WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 and 24 hours. Twenty micrograms of total liver lysates were used to measure 20S proteasome activity using a fluorogenic substrate. Results are presented as Means ± SE from 4 different mice.

3.4.6 Parkin KO Mice had Increased Proliferation Compared to WT Mice

Following APAP-induced cellular necrosis, it is well known that the liver has the capacity for repair and complete recovery if the level of injury is not too severe. Parkin KO mice have previously been shown to have increased proliferation leading to eventual development of hepatocellular carcinoma (HCC) at 18 months (Wang, Denison et al. 2004, Fujiwara, Marusawa et al. 2008). Therefore, we evaluated proliferation levels in WT and Parkin KO mice after APAP treatment to determine if greater levels of hepatocyte proliferation could be an additional mechanism of protection against APAP-induced liver injury in KO mice. Parkin KO mice had significantly increased basal expression levels of the proliferation proteins CyclinD1 and proliferating cell nuclear antigen (PCNA) compared to WT mice. In addition, Parkin KO mice had increased expression of these proliferative proteins compared to WT mice after APAP treatment for 6 hours as shown by CyclinD1 and PCNA western blot analysis (**Figure 3.4.6 A-B and E-F**). Immunohistochemistry for PCNA staining also revealed an increase in the number of PCNA positive cells in APAP-treated Parkin KO mice compared with APAP-treated WT mice, but these changes did not reach statistical significance (**Figure 3.4.6 C-D**). This was likely due to large variations in the immunohistochemistry analysis from the limited liver areas that we could assess in each sample, although we counted 6 random images from each sample. Nevertheless, these data suggest that increased proliferation levels in Parkin KO mice may provide protection against APAP-induced liver injury by allowing for faster replacement of necrotic hepatocytes and subsequent regeneration compared to WT mice.

Figure 3.4.6

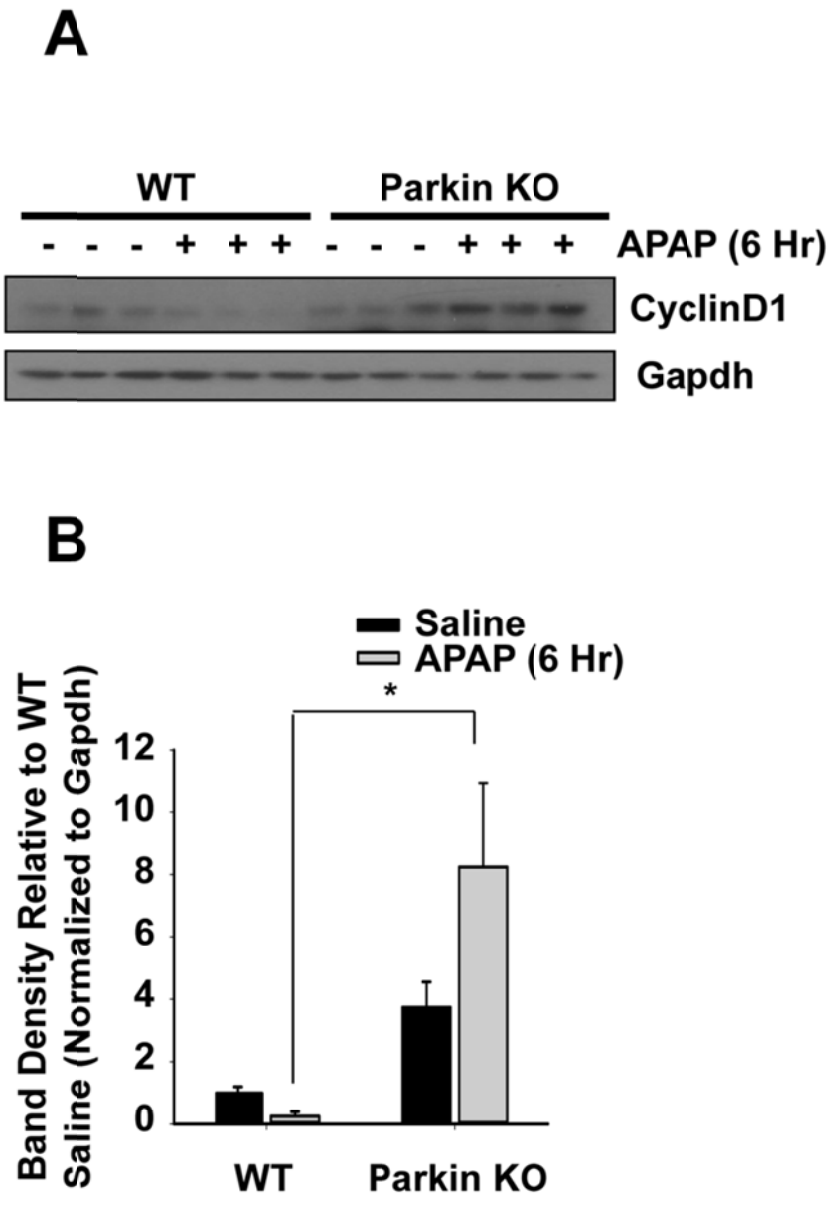
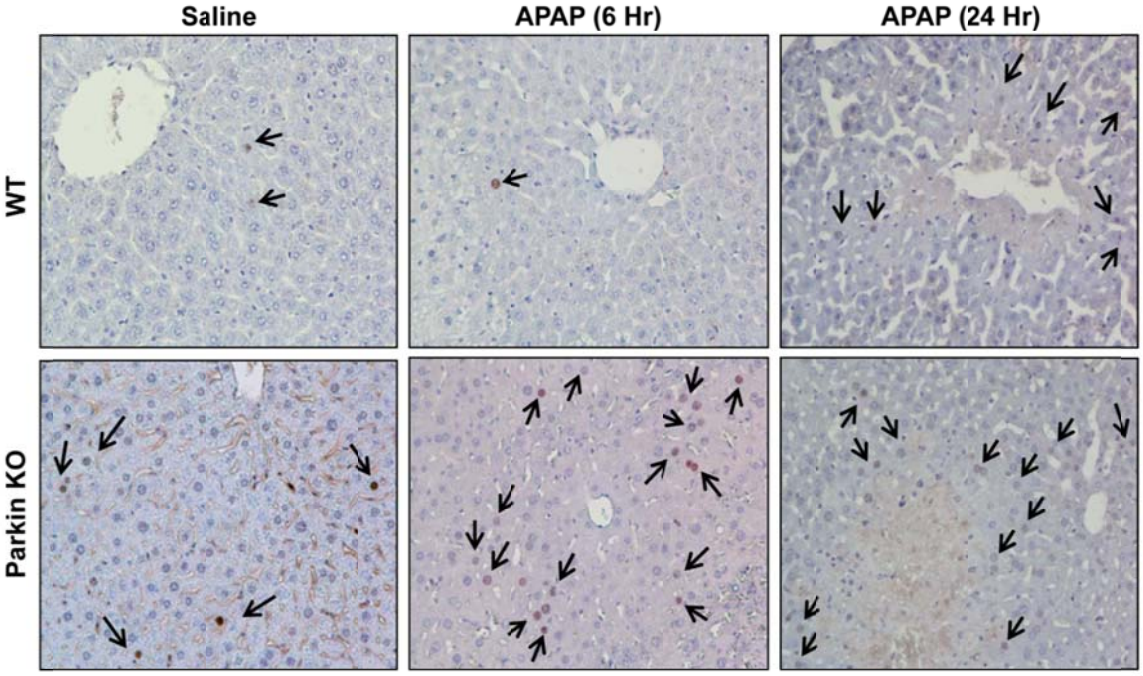

A
WT
Parkin KO
- - - + + + - - - + + +
APAP (6 Hr)
CyclinD1
Gapdh
B
Saline
APAP (6 Hr)
*
Band Density Relative to WT
Saline (Normalized to Gapdh)
12
10
8
6
4
2
0
WT
Parkin KO

Figure 3.4.6 Cont.

C



D

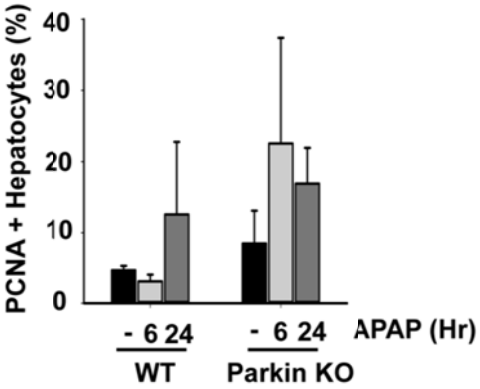


Figure 3.4.6 Cont.

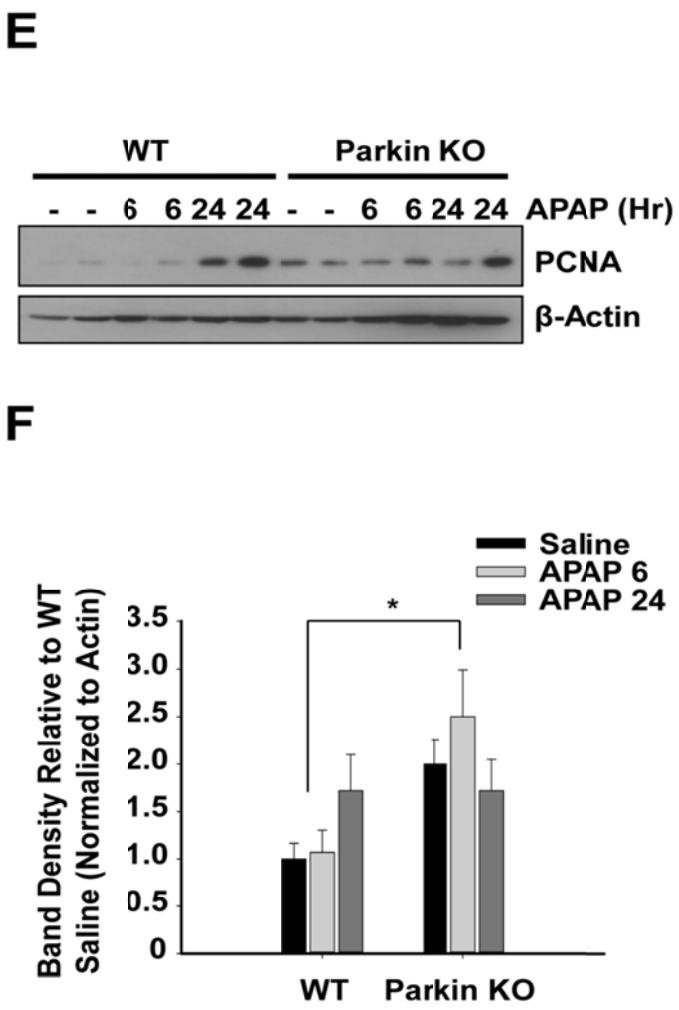


Figure 3.4.6: Protection in Parkin KO mice may be due to increased hepatocyte proliferation. A) WT and Parkin KO mice were treated with 500 mg/kg APAP or saline control for 6 hours, and total liver lysates were analyzed by western blot. **B)** Densitometry quantification of western blots for Cyclin D1. Data shown are Means ± SE (n=4 per group). Results were normalized to Gapdh. **C**) Representative images from PCNA staining are shown (arrows represent PCNA positive hepatocytes). **D)** PCNA staining quantification. Results are presented as % PCNA positive hepatocytes. Data shown are Means ± SE (n=3, 100x magnification, no significant differences between groups). Six images were quantified per liver tissue. **E**) Mice were treated as in **A**) and total liver lysates were analyzed by Western blot. **F)** Densitometry quantification of Western blots for PCNA. Data shown are Means ± SE (n=3-4 per group, *=p<0.05). Results were normalized to β-Actin.

3.4.7 APAP Treatment Induced Proteasomal Degradation of Mcl-1 and Necrosis in Cultured Mouse Hepatocytes Independent of Parkin

To further determine whether the proteasome plays a role in APAP-induced changes of Mcl-1, we treated primary cultured mouse hepatocytes with APAP in the presence or absence of Bortezomib (Bort), a proteasome inhibitor. Unlike the *in vivo* mouse liver, there was no difference in basal levels of Mcl-1 between the hepatocytes isolated from WT and Parkin KO mice. APAP treatment decreased Mcl-1 levels, which were recovered by Bort in both WT and Parkin KO hepatocytes. Bort treatment alone also increased Mcl-1 levels compared to control hepatocytes independent of Parkin (**Figure 3.4.7 A-B**). These results indicate that APAP-induced changes in Mcl-1 are mediated by the proteasome. Intriguingly, WT hepatocytes lost the expression of Parkin after 24 hours culture (**Figure 3.4.7 C**), which is a time point that we started treating hepatocytes with drugs including APAP in previous studies (Ni, Bockus et al. 2012). We found that APAP treatment for 8 hrs increased the number of cells with decreased mitochondrial membrane potential (**Figure 3.4.7 D-E**). Consistent with our previous findings, APAP treatment also increased necrotic cells in WT hepatocytes. Interestingly, we found that there was no difference in APAP-induced necrosis between WT and Parkin KO hepatocytes (**Figure 3.4.7 F-G**). Taken together, Parkin is dispensable for APAP-induced Mcl-1 degradation and necrosis in primary cultured mouse hepatocytes.

Figure 3.4.7

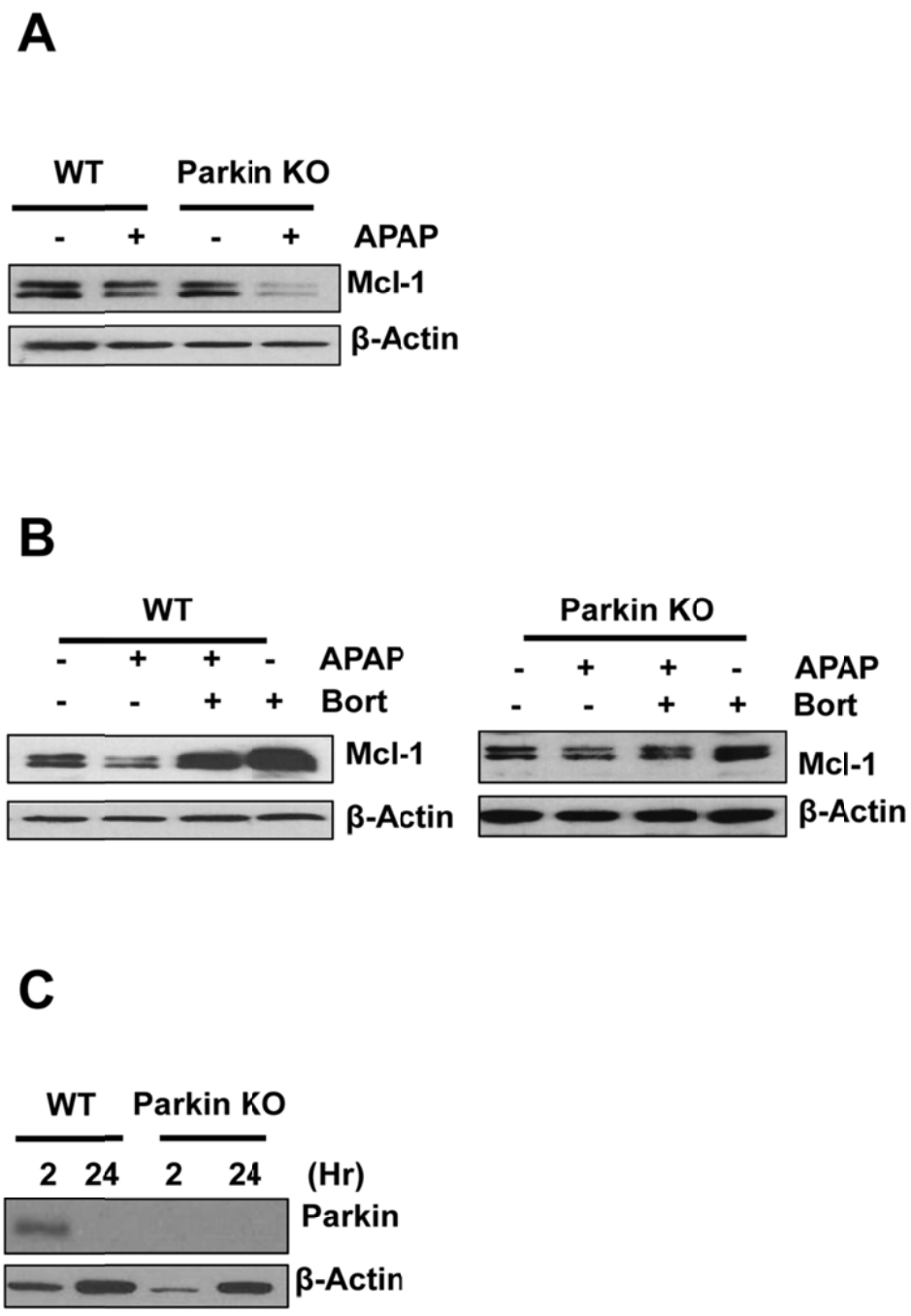

A
WT
Parkin KO
- + - + APAP
Mcl-1
β-Actin
B
WT
Parkin KO
- + + - APAP
- - + + Bort
Mcl-1
β-Actin
C
WT
Parkin KO
2 24 2 24 (Hr)
Parkin
β-Actin

Figure 3.4.7 Cont.

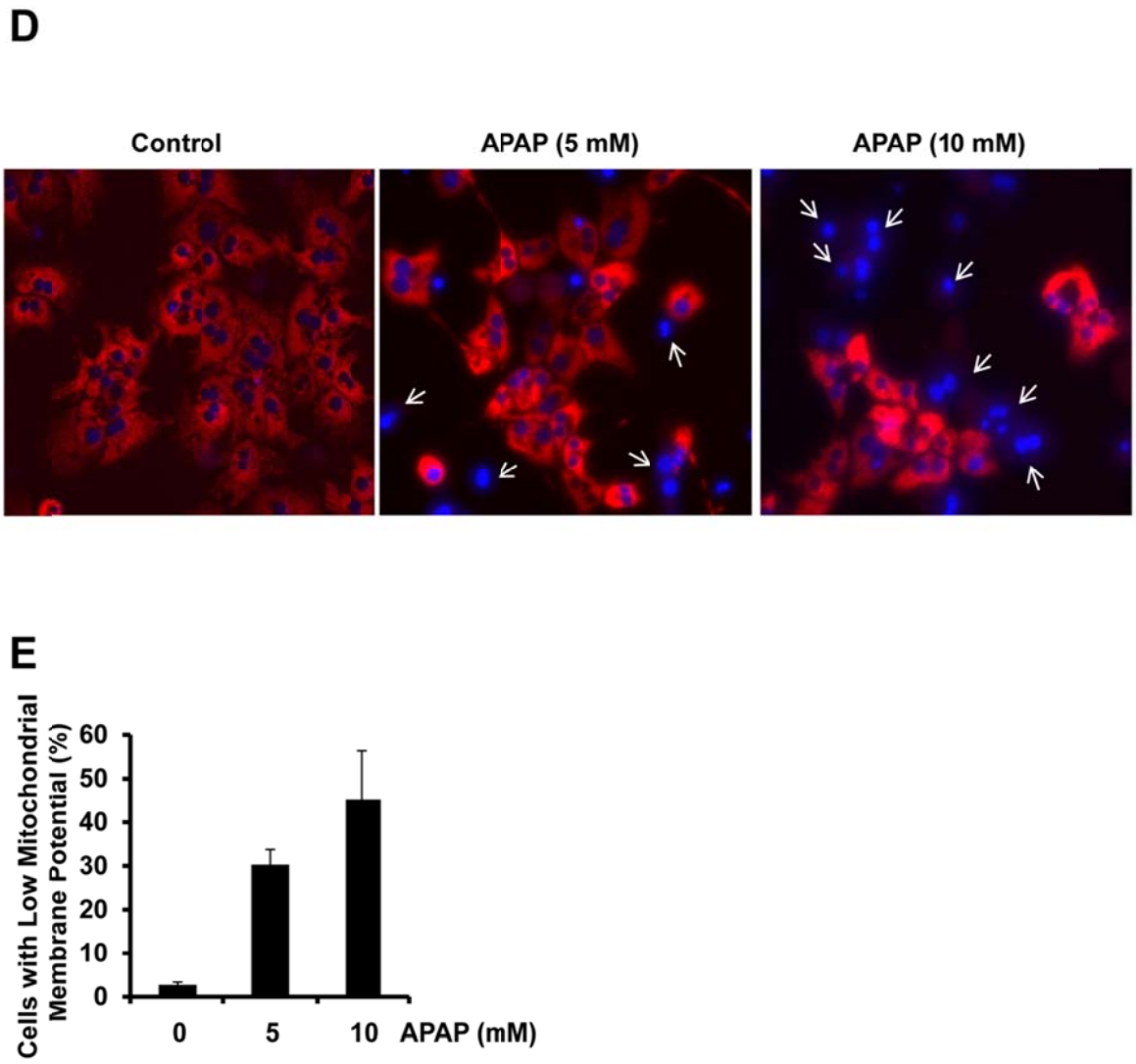


Figure 3.4.7 Cont.

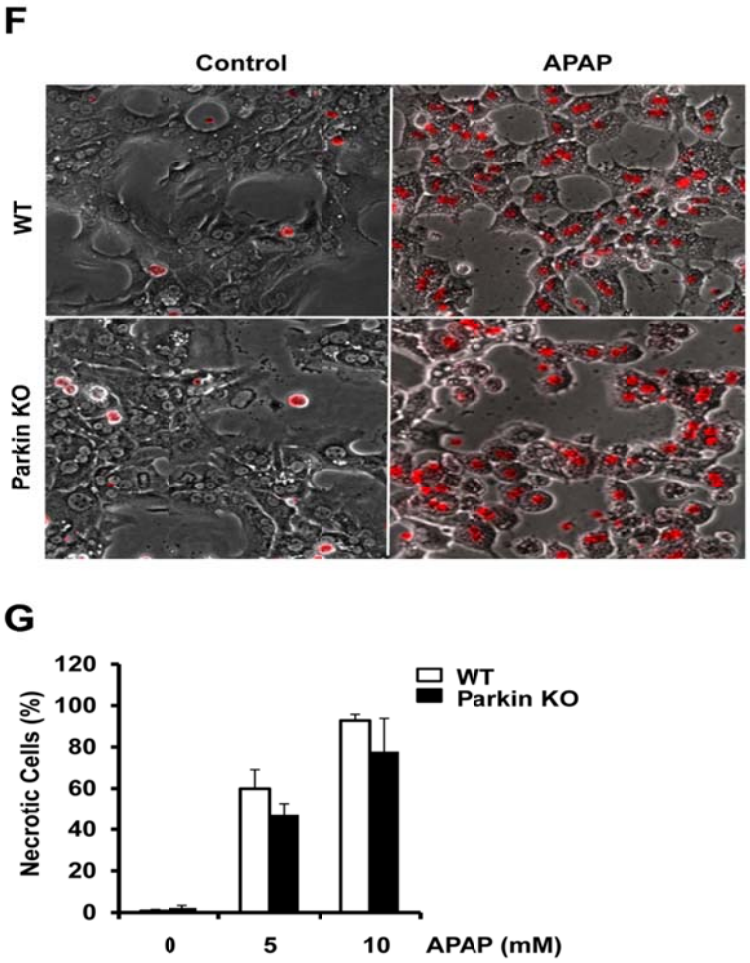


Figure 3.4.7: APAP induced Mcl-1 degradation and necrosis in cultured mouse hepatocytes independent of Parkin. **A-B**) Isolated hepatocytes from WT and Parkin KO mice were treated with APAP (10 mM) for 6 hours in the presence or absence of Bort (50 nM). Total cell lysates were subjected to Western blot analysis. **C**). Isolated hepatocytes from WT and Parkin KO mice were cultured for 2 and 24 hours. Total cell lysates were subjected to Western blot analysis for Parkin. Representative blots from 3 independent experiments are shown. **D**) Hepatocytes were first loaded with *TMRM* (50 nM) for 15 minutes and then treated with APAP for 8 hours. Cells were further stained with Hoechst 33342 (1 µg/mL) for 5 minutes followed by fluorescence microscopy. Representative overlayed images are shown. Arrows denote the cells with loss of mitochondrial membrane potential. **E**) The number of cells with loss of mitochondrial membrane potential was quantified. Data are Means ± SE from 3 independent experiments (More than 300 hundred cells were counted in each experiment from 3-4 different fields, *=$p<0.05$). **F**) WT and Parkin KO hepatocytes were treated with APAP for 24 hours, and cells were stained with PI (1 µg/mL) for 5 minutes followed by microscopy. Representative phase-contrast images overlayed with PI signals are shown. **G**) The percentage of PI positive cells was quantified. Data shown are Means ± SE from 3 independent experiments (at least three different images were randomly chosen from each experiment and more than 300 cells were counted, n.s.: no statistical significance).

3.4.8 Acute Knockdown of Parkin in Mouse Livers Impaired Mitophagy and Exacerbated APAP-induced Liver Injury

Since Parkin KO mice have no obvious phenotypes, it is possible that the mice may develop compensatory and adaptive mechanisms for the chronic loss of Parkin, which may contribute to their resistance to APAP-induced liver injury. To overcome the potential compensation and adaptation by the chronic loss of Parkin, we determined APAP-induced mitophagy and liver injury after acute knockdown of Parkin in mouse livers using an Ad-shRNA approach. We found that Ad-Parkin shRNA by tail vein injection dramatically reduced the expression of Parkin in mouse livers but had no effect on the expression of Parkin in mouse pancreas or skeletal muscle (**Figure 3.4.8 A**). In contrast to Parkin KO mice, mice given Ad-Parkin shRNA had increased serum ALT levels and hepatic necrosis compared with mice given Ad-Neg shRNA (**Figure 3.4.8 B-C**). Notably, mice that received adenovirus treatment had decreased sensitivity to APAP administration since ALT levels were less compared to the non-viral infected mice that were treated with the same dose of APAP (**Figure 3.4.8 B**). Our results are consistent with a previous study that showed reduced susceptibility to APAP after viral infection in mice (Getachew, James et al. 2010). There were no significant differences in Cyp2e1 or APAP-adduct levels between Ad-Parkin and Ad-Neg shRNA treated groups, but Ad-Parkin shRNA markedly decreased hepatic Parkin expression (**Figure 3.4.8 D**).

The Ad-Parkin shRNA-treated mice had increased phosphorylated levels of JNK but decreased levels of Mcl-1 and PCNA compared to Ad-Neg shRNA-treated mice after administration of APAP (**Figure 3.4.8 E**). The levels of LC3-II, a marker for autophagy, were increased after APAP treatment in both Ad-Neg and Ad-Parkin-treated mice (**Figure 3.4.8 E**), suggesting that APAP increased the numbers of autophagosomes/autolysosomes independent of Parkin. Moreover, the number of mitophagosomes was significantly decreased in mouse livers treated with Ad-Parkin shRNA compared with Ad-Neg shRNA after administration of

APAP (**Figure 3.4.8 F-G**). These results suggest that acute knockdown of Parkin in mouse livers had impaired mitophagy and hepatocyte proliferation, decreased hepatic Mcl-1 expression and increased JNK activation, which exacerbated liver injury in response to APAP administration. Consistent with the LC3-II results from Western blot analysis, the number of total autophagosomes was very comparable between groups of Ad-Parkin shRNA and Ad-Neg shRNA after administration of APAP (**Figure 3.4.8 H**), suggesting that acute knockdown of Parkin does not affect general autophagy. These results suggest that acute knockdown of Parkin in mouse livers impaired mitophagy and hepatocyte proliferation, decreased hepatic Mcl-1 expression and increased JNK activation, which exacerbated liver injury in response to APAP administration.

Figure 3.4.8

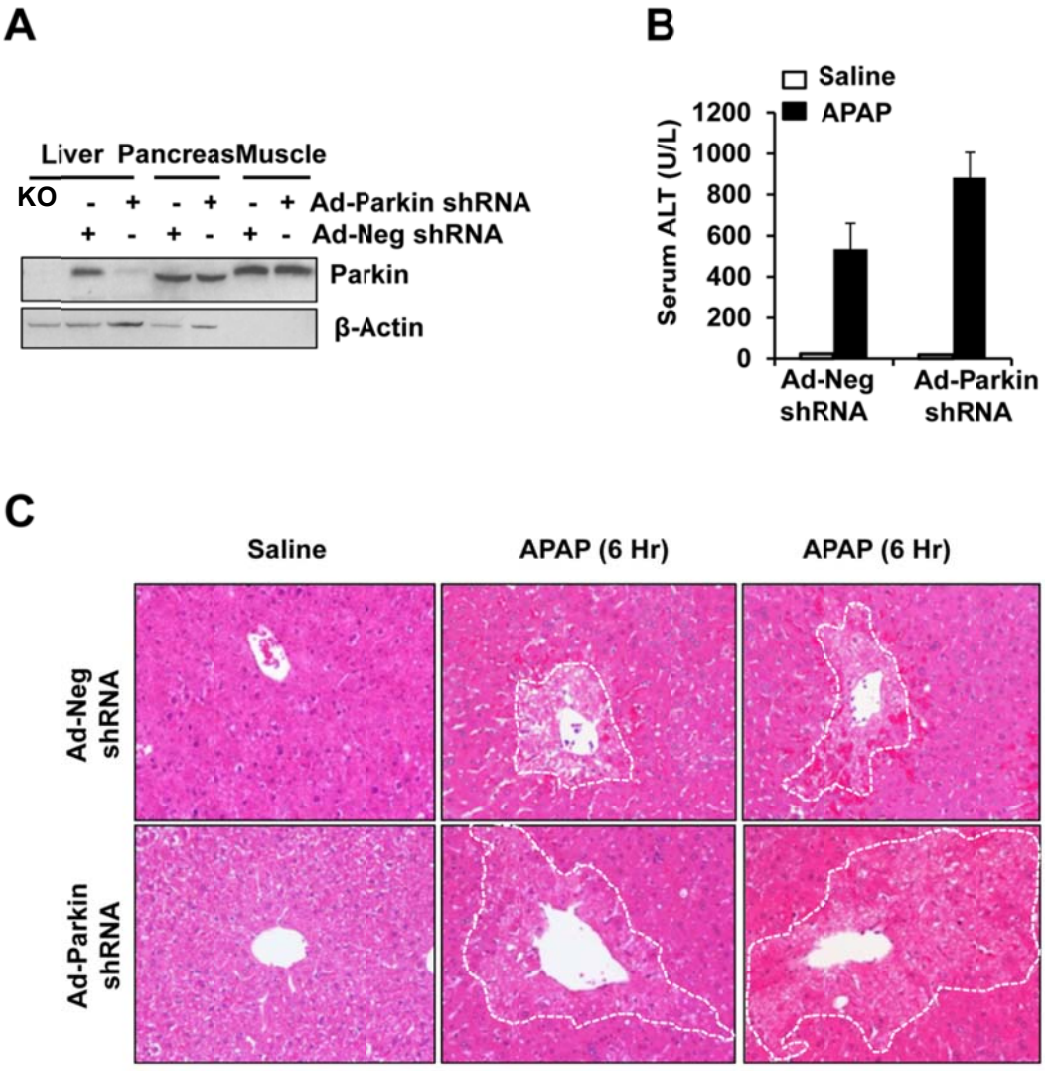
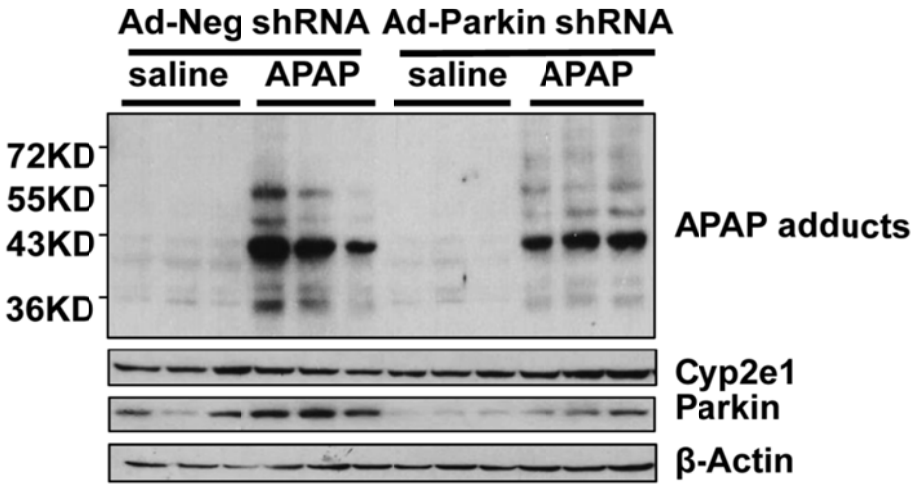

A
Liver Pancreas Muscle
KO - + - + - + Ad-Parkin shRNA
+ - + - + - Ad-Neg shRNA
Parkin
β-Actin
B
Saline
APAP
Serum ALT (U/L)
1200
1000
800
600
400
200
0
Ad-Neg shRNA
Ad-Parkin shRNA
C
Saline
APAP (6 Hr)
APAP (6 Hr)
Ad-Neg shRNA
Ad-Parkin shRNA

Figure 3.4.8 Cont.

D



E

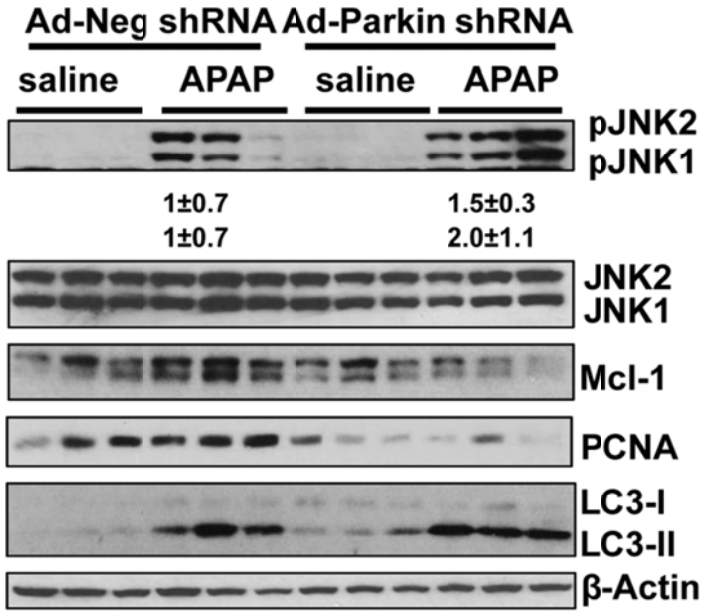


Figure 3.4.8 Cont.

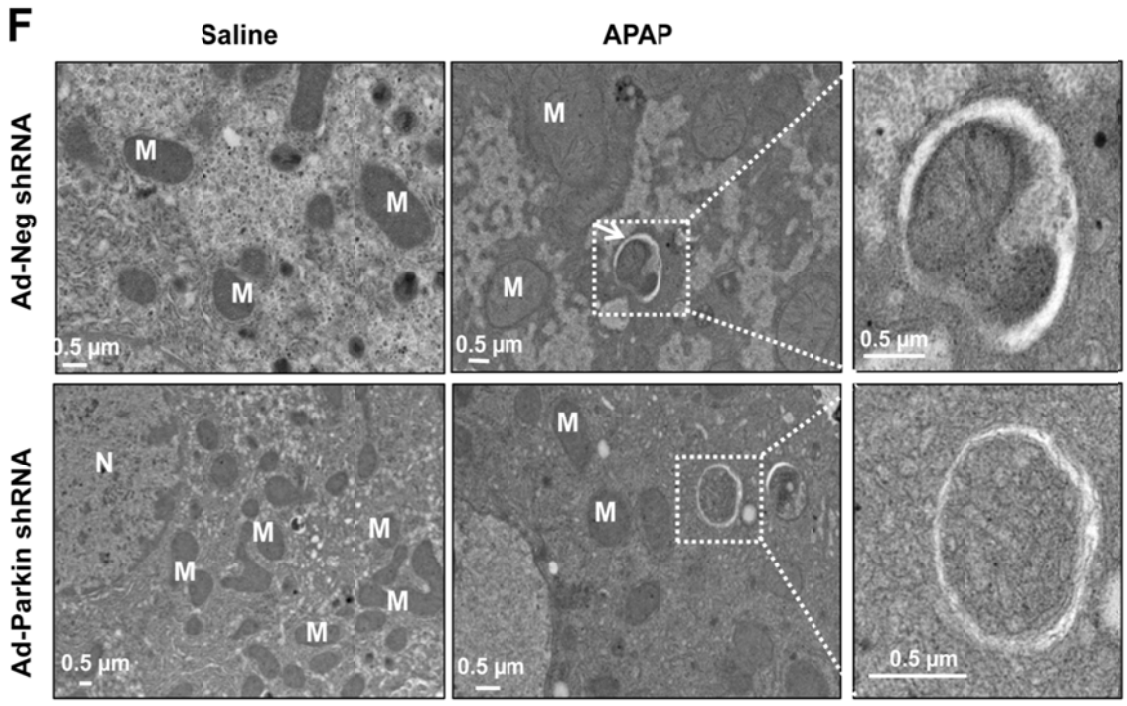


Figure 3.4.8 Cont.

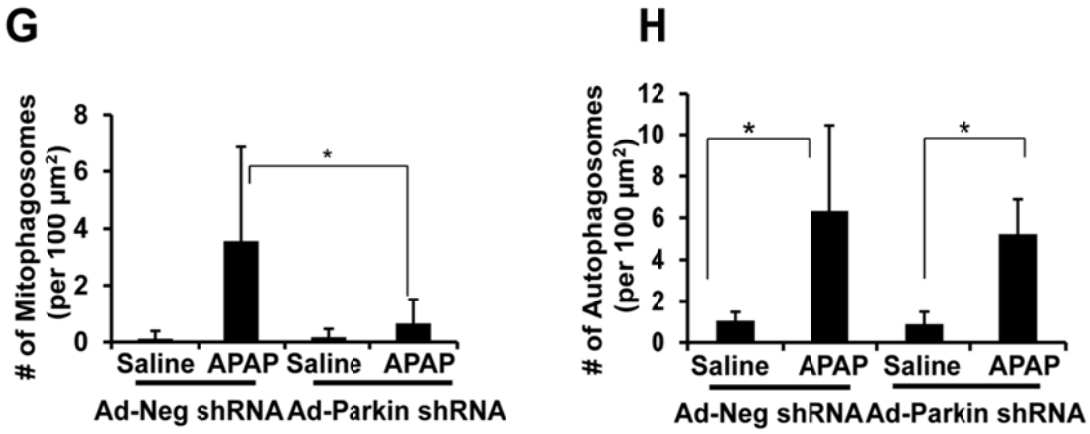


Figure 3.4.8: Acute knockdown of Parkin in mouse livers impaired APAP-induced mitophagy and exacerbated APAP-induced liver injury. **A**) Male C57Bl/6J mice were treated either with Ad-Neg or Ad-Parkin shRNA (iv., 1×10^9 PFU per mouse) for 4 days. Total liver, pancreas and skeletal muscle lysates were subjected to western blot analysis. Lysate from a Parkin KO mouse liver was used a negative control. Mice were further treated with APAP (500 mg/kg) or saline for another 6 hours. **B**) Blood samples were used to measure serum ALT levels. Data shown are Means±SE (n=4, *=p<0.05). **C**) Representative H&E images are shown (200x magnification).Dot line circled areas denote centrilobular necrosis**. D-E)** Total liver lysates were subjected to western blot analysis. Densitometry quantification of western blots for P-JNK1 and P-JNK2 (n=3 per group). Results were normalized to β-Actin. **F**) Representative EM images are shown. An enlarged image of a mitophagosome showing an enveloped mitochondrion and an enlarged image of an autophgosome showing enveloped cytosolic proteins are from the dotted line boxed areas (arrow=mitophagosomes, M=mitochondria, N=nucleus). **G-H)** Quantification of EM images for mitophagosomes and total autophagosomes. Data shown are Means±SE (n $\geq$ 20 images quantified per group, *=p<0.05).

3.5 Discussion

In this study, we found that APAP treatment induced Parkin translocation to mitochondria and increased levels of mitochondrial protein ubiquitination in WT mouse livers. However, we found that Parkin differentially regulated mitophagy and APAP-induced liver injury depending on whether Parkin was chronically deleted in mice or acutely knocked down in mouse livers. We found that mitophagy still occurred in Parkin KO mouse livers after APAP treatment, suggesting that Parkin may be dispensable for mitophagy under chronic Parkin loss conditions. Moreover, we surprisingly found that Parkin KO mice were protected against APAP-induced liver injury compared to WT mice. In contrast, mice with acute knockdown of Parkin had decreased hepatic mitophagy and increased APAP-induced liver injury.

3.5.1 Parkin was Dispensable for APAP-induced Mitophagy in Parkin KO Mice but not in Mice with Acute Knockdown of Hepatic Parkin

Parkin translocated to mitochondria after APAP treatment in WT mice, which correlated with an increase in mitochondrial protein ubiquitination, suggesting that Parkin-induced mitophagy occurs in mouse liver after APAP treatment. However, we found Parkin KO mice had similar mitochondrial protein degradation after APAP treatment, although there was a slight reduction in the number of mitophagosomes in Parkin KO mice compared to WT mice. Mitophagy is likely a protective mechanism against APAP-induced liver injury by promoting removal of damaged mitochondria in both WT and Parkin KO mice. However, it appears that Parkin is not required for mitophagy induction in the liver because mitophagy still occurred in Parkin KO mouse livers after APAP treatment. In contrast, mice with acute knockdown of Parkin had significantly reduced mitophagy after APAP administration. These results suggest that alternative compensatory mechanisms for mitophagy independent of Parkin may occur when Parkin is chronically deleted in mice after APAP administration. To support this notion, it was

previously reported that compensatory mitophagy also occurred in Parkin KO mouse hearts (Piquereau, Godin et al. 2013). It is likely that compensatory and adaptive alternative mechanisms for mitophagy independent of Parkin cannot be established within the relative short time period after acute Parkin knockdown. Alternatively, although it seems to be less likely, the loss of Parkin in other tissues in Parkin KO mice may also affect the mitophagy signaling pathway in the liver. The tissue cross-talk regulating mitophagy in the liver is currently unknown.

It was recently reported that at least three distinct types of mitophagy could occur in hepatocytes: 3-methyladenine (3-MA) sensitive, 3-MA insensitive and mitochondrial derived vesicles (Czaja, Ding et al. 2013, Lemasters 2014). We previously showed that 3-MA inhibited APAP-induced autophagy and enhanced APAP-induced necrosis in cultured mouse hepatocytes (Ni, Bockus et al. 2012). These results suggest that APAP may induce 3-MA sensitive mitophagy. In addition to canonical mitophagy, we also showed that APAP could induce the formation of mitochondrial spheroids, a process of mitochondria remodeling that forms unique vesicle-like structures derived directly from mitochondria in mouse livers (Ding, Guo et al. 2012, Ni, Williams et al. 2013).

The identification of mitophagy in Parkin KO mice after APAP administration suggests that Parkin may not be essential for mitophagy induction *in vivo*. The mechanism for alternative Parkin-independent mitophagy induction in the liver is currently unknown but could be due to upregulation of other important proteins or pathways in the absence of Parkin that have been shown to have a role in the mitophagy pathway (discussed in section 5.1.1). For example, Parkin-independent mitophagy in Parkin KO mice may be mediated by another E3 ubiquitin ligase, such as March5, Smurf1, or Mulan. In addition, Parkin-independent mitophagy may occur via Bcl2/adenovirus E1B 19kDa interacting protein 3 (Bnip3), Fun14 domain containing 1 (Fundc1), or Nix. Bnip3 and Fundc1 have both been shown to play a role in mitophagy induction for removal of damaged mitochondria to prevent accumulation of reactive oxygen species

during hypoxia (Zhang, Bosch-Marce et al. 2008, Liu, Feng et al. 2012). Nix also plays a role in selective removal of mitochondria during red blood cell maturation (Schweers, Zhang et al. 2007, Sandoval, Thiagarajan et al. 2008, Zhang and Ney 2008). In addition, Nix has been shown to play a role in Parkin-dependent mitophagy induction by promoting mitochondrial depolarization and translocation of Parkin to mitochondria (Ding, Ni et al. 2010). Cardiolipin, a phospholipid located on the inner mitochondrial membrane, has also been recently shown to have a role in mitophagy induction in neurons by translocating to the outer mitochondrial membrane and interacting with LC3 (Chu, Ji et al. 2013, Chu, Bayir et al. 2014). Therefore, cardiolipin may also have a role in Parkin-independent mitophagy induction in the liver. Even though the mechanism of Parkin-independent mitophagy induction in the liver is not currently understood, induction of mitophagy in both WT and Parkin KO mouse livers was likely a mechanism of protection against APAP-induced liver injury by removing damaged mitochondria. However, in addition to mitophagy, Parkin KO mice had other protective mechanisms against APAP-induced liver injury and necrosis compared to WT mice, which are further discussed below.

3.5.2 JNK Activation was Differentially Regulated in Parkin KO and Acute Parkin Knockdown Mice after APAP Treatment

Another mechanism of protection in Parkin KO mice was decreased JNK activation compared to WT mice after APAP treatment. JNK activation, particularly in the mitochondria, has been shown to exacerbate APAP-induced liver injury (Gunawan, Liu et al. 2006, Latchoumycandane, Goh et al. 2007, Hanawa, Shinohara et al. 2008, Saito, Lemasters et al. 2010). Parkin has been previously shown to negatively regulate JNK activity in *Drosophila* (Cha, Kim et al. 2005, Hwang, Kim et al. 2010). Parkin-mutant flies contained high levels of phosphorylated JNK in their dorsomedial neurons, resulting in shrinkage of the neuronal cell

body, which was not present in control flies. Neuron cell body morphology was normal when Parkin-mutant flies were treated with a dominant negative form of JNK, confirming that the morphology changes were caused by increased JNK activation in the absence of Parkin (Cha, Kim et al. 2005). Parkin was also found to suppress JNK activation in the *Drosophila* eye during development by transcriptionally repressing *basket (bsk)*, which is a gene encoding for JNK (Hwang, Kim et al. 2010). In addition, Parkin was shown to suppress JNK activation *in vitro* in EPP85 human pancreatic carcinoma cells via mono-ubiquitination of heat-shock protein 70 (HSP70), but the exact mechanism for how mono-ubiquitinated HSP70 inhibits activation of JNK is unknown (Liu, Aneja et al. 2008). Furthermore, Parkin was shown to inhibit JNK activity in COS1 monkey kidney fibroblasts (Cha, Kim et al. 2005). Parkin and JNK were co-transfected into COS1 cells, and co-expression of Parkin reduced JNK activation (Cha, Kim et al. 2005). Consistent with these findings, we found that APAP increased JNK activation in acute Parkin knockdown mouse livers. In contrast, JNK activation was decreased in Parkin KO mouse livers compared to WT mice after APAP treatment. These results suggest that acute knockdown and chronic loss of Parkin differentially impact JNK activation, which is likely due to induction of some adaptive mechanisms in the mouse livers when Parkin is chronically absent.

The adaptive mechanisms for JNK inhibition after APAP treatment in Parkin KO mice is currently unknown but may involve mono or polyubiquitination of upstream or downstream mediators of JNK activation. For example, chronic loss of Parkin may affect the degradation of MAP kinase phosphatase-1 (MkP-1), an endogenous inhibitor of JNK activation (Doddareddy, Rawling et al. 2012). Parkin has also been shown to stabilize proteins via monoubiquitination, so chronic loss of Parkin may also stabilize upstream activators of JNK such as Apoptosis signal-regulating kinase 1 (Ask1) via monoubiquitination (Dunn, Wiltshire et al. 2002). Taken together, it appears that chronic and acute Parkin loss have contrasting impacts on JNK

activation in mouse livers after APAP treatment, which may subsequently protect against or exacerbate APAP-induced liver injury.

3.5.3 Mcl-1 Expression was Differentially Regulated in Parkin KO and Acute Parkin Knockdown Mice after APAP Treatment

Mcl-1 is an anti-apoptotic Bcl-2 protein that is mostly localized to the mitochondrial membrane (Warr and Shore 2008), and its stabilization has been shown to play a protective role against APAP-induced hepatocellular necrosis (Sharma, Gadang et al. 2012). Parkin KO mice had increased liver Mcl-1 expression compared to WT mice before and after APAP treatment, which may be another mechanism for their protection against APAP-induced liver injury. APAP has been shown to induce degradation of Mcl-1 in the early phase of liver injury in starved mice (1 to 4 hours after treatment) (Shinohara, Ybanez et al. 2010), but we did not observe degradation of Mcl-1 in WT or Parkin KO mice after APAP treatment in fed mice. We actually observed an increase in Mcl-1 expression after APAP treatment. This discrepancy could be due to several possibilities. First, we used fed mice to avoid the possible interference of starvation-induced autophagy whereas Shinohara *et al*. used starved animals. Starvation may inactivate AKT and interfere with AKT-GSK3β-mediated Mcl-1 phosphorylation and proteasomal degradation. Consistent with this notion, we also found that APAP decreased Mcl-1 in primary cultured hepatocytes in serum free medium. Second, we assessed APAP-induced liver injury 6 hours after treatment, which may allow for recovery of Mcl-1 protein levels. Third, Mcl-1 expression is regulated by Gsk-3β phosphorylation, which primes it for ubiquitination and degradation by the proteasome (Maurer, Charvet et al. 2006). Gsk-3β is known to translocate to mitochondria after APAP-induced liver injury where it phosphorylates Mcl-1 to initiate its proteasomal degradation (Shinohara, Ybanez et al. 2010). Since phosphorylation of GSK-3β at serine 9 normally inhibits its activity (Doble and Woodgett 2003), increased serine 9

phosphorylation of GSK-3β may thus contribute to the increased Mcl-1 levels following APAP treatment. Fourth, Parkin KO mice tend to have decreased proteasome activity compared to WT mice, which may also partially contribute to increased Mcl-1 protein expression compared to WT mice. In primary cultured mouse hepatocytes, Parkin seems to be less important in regulating Mcl-1 levels after APAP treatment since WT hepatocytes lost the expression of Parkin during culture and APAP decreased Mcl-1 protein levels in both cultured WT and Parkin KO hepatocytes. The decreased Mcl-1 protein levels were reversed by a proteasome inhibitor, supporting the important role of the proteasome in regulating Mcl-1 levels in APAP-treated hepatocytes. Finally, Mcl-1 degradation has also been shown to be induced by JNK phosphorylation in HeLa cells (Wang, Wang et al. 2014). Parkin KO mice had reduced JNK activation, which may be linked to their increased Mcl-1 expression. We found that acute knockdown of Parkin in mouse livers had decreased Mcl-1 protein levels after APAP treatment. Interestingly, Parkin knockdown mice also had increased JNK activation in their livers after APAP treatment, which is opposite from APAP-treated Parkin KO mouse livers. These findings are also in line with the known role of JNK in regulating Mcl-1. In addition, the mTOR pathway has been shown to have a role in Mcl-1 transcriptional regulation in Parkin KO mouse neurons because neurons in Parkin KO mice had increased Mcl-1 expression due to compensatory activation of mTOR (Ekholm-Reed, Goldberg et al. 2013). Future work is needed to determine the exact role of JNK and mTOR in regulating Mcl-1 during APAP overdose.

3.5.4 Increased Hepatocyte Proliferation in Parkin KO Mice, but not in Mice with Acute Knockdown of Parkin, after APAP Treatment

Parkin KO mice had increased basal hepatocyte proliferation levels and increased hepatocyte proliferation after APAP treatment for 6 and 24 hours, which may provide protection against APAP-induced hepatocellular necrosis and liver injury by allowing for faster replacement of necrotic hepatocytes. Parkin has been previously shown to be a tumor suppressor in liver, and Parkin KO mice develop spontaneous liver tumors around 18 months of age (Fujiwara, Marusawa et al. 2008). In addition, Parkin expression is significantly decreased or absent in most cancer cell lines and human liver tumors (Wang, Denison et al. 2004). This tumor suppressive function of Parkin was likely important in protection against APAP-induced liver injury, which causes significant hepatocellular necrosis. Hepatocytes undergoing division were shown to be protected against carbon tetrachloride due to increased basal hepatocellular proliferation (Mehendale, Thakore et al. 1994). Therefore, with increased basal proliferation and increased proliferation after APAP treatment compared to WT mice, Parkin KO mice were likely able to replace their necrotic hepatocytes faster than WT mice, making them resistant to APAP-induced hepatocellular necrosis and liver injury. In contrast, acute knockdown of Parkin had decreased hepatocyte proliferation with or without APAP treatment. Our results suggest that acute and chronic loss of Parkin have different impacts on hepatocyte proliferation, and in turn differentially affect APAP-induced liver injury.

3.6 Conclusions

In conclusion, Parkin-induced mitophagy is likely a mechanism of protection against APAP-induced liver injury in WT mice because Parkin translocated to mitochondria after APAP treatment. However, Parkin is dispensable for APAP-induced mitophagy in Parkin KO mouse livers. Parkin KO mice may inhibit APAP-induced liver injury by attenuating APAP-induced JNK activation and Mcl-1 degradation while also increasing hepatocyte proliferation. In addition, currently unidentified Parkin-independent mitophagy pathways activated as a compensatory and adaptive response during the chronic loss of Parkin likely also play a protective role against APAP-induced liver injury by removing damaged mitochondria. These protective effects in Parkin KO mice were reversed in mice with acute knockdown of Parkin. Therefore, chronic deletion (KO) and acute knockdown of Parkin differentially regulate APAP-induced mitophagy and liver injury in mice. Our results also suggest that caution needs to be exercised for data interpretation when using genetic KO mice for assessing drug-induced liver injury.

Chapter 4: Parkin Regulates Mitophagy and Mitochondrial Function to Protect Against Alcohol-induced Liver Injury and Steatosis in Mice

4.1 Abstract

Alcoholic liver disease claims two million lives per year. We previously reported that autophagy protected against alcohol-induced liver injury and steatosis by removing damaged mitochondria. However, the mechanisms for removal of these mitochondria are unknown. Parkin is an evolutionarily conserved E3 ligase that is recruited to damaged mitochondria to initiate ubiquitination of mitochondrial outer membrane proteins and subsequent mitochondrial degradation by mitophagy. In addition to its role in mitophagy, Parkin has been shown to have other roles in maintaining mitochondrial function. We investigated if Parkin protected against alcohol-induced liver injury and steatosis using Wild-type (WT) and Parkin knockout (KO) mice treated with alcohol by the acute-binge and Gao-binge (chronic plus acute-binge) models. We found that Parkin protected against liver injury in both alcohol models, likely due to Parkin's role in maintaining a population of healthy mitochondria. Alcohol caused greater mitochondrial damage and oxidative stress in Parkin KO livers compared to WT livers. Parkin KO mice had severely swollen and damaged mitochondria that lacked cristae after alcohol treatment, which were not seen in WT mice. Furthermore, Parkin KO mice had decreased mitophagy, β-oxidation, mitochondrial respiration, and cytochrome c oxidase activity after acute alcohol treatment compared to WT mice. Interestingly, liver mitochondria seemed able to adapt to alcohol treatment, but Parkin KO mouse liver mitochondria had less capacity to adapt to Gao-binge treatment compared to WT mouse liver mitochondria. Overall, our findings indicate that Parkin is an important mediator of protection against alcohol-induced mitochondrial damage, steatosis, and liver injury.

4.2 Introduction

Alcoholic liver disease (ALD) (discussed in section 1.7) is a health problem worldwide that claims two million lives per year (Rehm, Samokhvalov et al. 2013), and 50% of people in the United States over the age of 18 consume alcohol regularly (Nassir and Ibdah 2014). ALD pathogenesis begins with liver steatosis, which is reversible with abstinence from alcohol. However, with continuous alcohol abuse, ALD can progress in some patients from steatosis to steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma with prolonged alcohol abuse. Mechanisms for progression of ALD pathogenesis are still not completely understood. In addition, there is currently no cure for ALD other than liver transplantation in severe disease states (Gao and Bataller 2011, Williams, Manley et al. 2014). Therefore, a better understanding of mechanisms involved in ALD pathogenesis is greatly needed for future generation of therapeutics to combat liver disease caused by alcohol.

Macroautophagy (hereafter referred to as autophagy) (discussed in section 1.2) is a protective process that initiates lysosomal degradation of cellular components. Autophagy is activated to provide the cell with nutrients during starvation or to remove damaged organelles and protein aggregates to prevent cell death and tissue injury. Autophagy occurs through formation of double-membrane autophagosomes, which engulf organelles and protein aggregates and shuttle them to the lysosome for degradation (Parzych and Klionsky 2014). We and others have previously shown that autophagy is protective against alcohol-induced liver injury (Ding, Li et al. 2010, Ding, Li et al. 2011, Lin, Zhang et al. 2013). Specifically, we found that autophagy protected against alcohol-induced liver injury and steatosis by selectively removing damaged mitochondria and lipid droplets by mitophagy and lipophagy, respectively (Ding, Li et al. 2010, Ding, Li et al. 2011). Mitophagy (discussed in section 1.3) has been shown to be activated as a protective mechanism by many different cellular stress conditions including loss of mitochondrial membrane potential, cellular reactive oxygen species (ROS), mitochondrial

DNA damage, and mitochondrial accumulation of aggregated proteins (Liu, Sakakibara et al. 2014), Some of these stresses, such as cellular ROS, nitric oxide and mitochondria DNA damage, have been associated with ALD pathogenesis (Zelickson, Benavides et al. 2011, Garcia-Ruiz, Kaplowitz et al. 2013, Andringa, Udoh et al. 2014) and likely lead to alcohol-induced mitophagy activation in the liver (King, Swain et al. 2014). However, mechanisms for alcohol-induced activation of mitophagy in the liver are currently unknown.

Mitophagy has been shown to require Parkin in *in vitro* models (discussed in section 1.3) (Narendra, Tanaka et al. 2008, Chan, Salazar et al. 2011, Ding, Guo et al. 2012). Parkin is an evolutionarily conserved E3 ligase that is recruited to damaged mitochondria by phosphatase and tensin homolog-induced putative kinase 1 (Pink1) to initiate ubiquitination of mitochondrial outer membrane proteins and subsequent mitochondrial degradation by mitophagy (Kawajiri, Saiki et al. 2010, Matsuda, Sato et al. 2010, Narendra, Jin et al. 2010). Parkin is mainly known for its protective role in the brain because loss of Parkin has been linked to autosomal recessive Parkinsonism (Kitada, Asakawa et al. 1998), but we found that Parkin is also expressed in liver (Ding and Yin 2012). The role of Parkin-induced mitophagy *in vivo* is not completely understood. We recently demonstrated that Parkin translocated to liver mitochondria after acetaminophen treatment in mice. In addition, mitophagy levels were decreased in Parkin knockout (KO) mice compared to WT mice after acetaminophen treatment, suggesting that removal of damaged mitochondria by Parkin-induced mitophagy is likely a protective mechanism in the liver (Williams, Ni et al. 2015). In addition to its role in mitophagy, Parkin has also been shown to help maintain mitochondrial function in the brain. Parkin KO mouse brain mitochondria had decreased mitochondrial respiration and dysregulation of proteins involved in energy metabolism and respiration with aging (Palacino, Sagi et al. 2004, Periquet, Corti et al. 2005, Stichel, Zhu et al. 2007). Therefore, we investigated the role of Parkin in mitophagy induction and in maintenance of mitochondrial function as protective mechanisms against alcohol-induced

liver injury using WT and Parkin KO mice. Two mouse alcohol models were used in this study: acute-binge and Gao-binge (chronic plus acute-binge). The acute-binge model represents human binge drinking, which is defined as consumption of greater than 4 or 5 drinks in a two-hour period for women and men, respectively (Shukla, Pruett et al. 2013). The acute-binge model is best for studying initial phases of ALD because it causes slight liver injury, steatosis, oxidative stress, and mitochondrial damage and dysfunction (Shukla, Pruett et al. 2013, Mathews, Xu et al. 2014). The Gao-binge model is thought to better reflect human disease because most chronic alcohol abusers also binge drink, and binge drinking in chronic alcohol abusers is thought to further progression from alcohol-induced steatosis to more severe liver pathologies, such as steatohepatitis. In addition, the Gao-binge alcohol model produces greater amounts of liver injury and steatosis than the acute-binge model while also producing mitochondrial damage and oxidative stress (Bertola, Mathews et al. 2013, Bertola, Park et al. 2013, Mathews, Xu et al. 2014, Williams, Manley et al. 2014).

We found that Parkin KO mice had increased liver injury, oxidative stress, and steatosis after alcohol treatment compared to WT mice. Parkin KO mouse livers had decreased mitophagy, β-oxidation, mitochondrial respiration, and cytochrome c oxidase (COX) activity compared to WT mouse livers after acute-binge alcohol treatment. Decreased hepatic mitochondrial function in Parkin KO mice was likely due to increased alcohol-induced mitochondrial damage and reduced mitophagy compared to WT mice. Interestingly, liver mitochondria seemed able to adapt to Gao-binge treatment, but mitochondria from Parkin KO mouse livers seemed less able to adapt to alcohol compared to WT mouse mitochondria resulting in severely damaged and swollen mitochondria in Parkin KO mouse livers. Our findings indicate that Parkin is an important protector against alcohol-induced liver injury and steatosis due to its roles in maintaining mitochondrial integrity and function after alcohol treatment.

4.3 Materials and Methods

Materials. Two hundred Proof ethanol (DSP-MD.43), maltose dextran (3653), and the Lieber-DeCarli ’82 Shake and Pour Liquid Control (F1259SP) and Ethanol (F1258SP) diets were all purchased from Bio-Serv. The kit used for alanine aminotransferase (ALT) measurement was purchased from Pointe Scientific (A7526-450). The following antibodies were used for western blot analysis: anti-Cyp2e1 (Abcam, ab19140), anti-β-Actin (Sigma, A5441), anti-Fasn (cell Signaling, 3180S), anti-Acc (Cell Signaling, 3676S), anti-Parkin (Santa-Cruz, SC-32282), anti-GAPDH (Cell Signaling, 2118), anti-Tom20 (Santa-Cruz, SC11415), anti-p62 (Abnova, H00008878-M01), Anti-HSP60 (Santa Cruz, sc-13115), anti-VDAC (Calbiochem, 529534), and anti-Cox II (Mitoscience, D1203). The Anti-LC3 antibody was generated as previously described (Ding, Li et al. 2010).

Animal Experiments. Male WT C57BL/6J and whole body Parkin KO mice (C57BL/6J background, #006582) were purchased from the Jackson Laboratory. All animals received humane treatment, and all protocols were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Eight to twelve week old male mice were treated with ethanol using one of two models: acute-binge (Ding, Li et al. 2010) or Gao-binge (Bertola, Mathews et al. 2013, Williams, Manley et al. 2014). For acute-binge, mice were fasted for 6 hours in the morning and then given ethanol (4.5 g/kg body weight) or an equivalent volume of water by oral gavage, which was spaced over 4 gavages spread 15 minutes apart. Mice were sacrificed 16 hours after binge. For Gao-Binge, mice were acclimated to the Lieber-DeCarli liquid control diet for 5 days followed by further feeding with the liquid control or ethanol diet (5%) for 10 days. The mice were then given an 8 hour ethanol (5 g/kg body weight) or maltose dextran (9 g/kg body weight) binge by oral gavage on the morning of day 16 (day 11 after the start of alcohol diet feeding). Gavages for Gao-binge were spaced over 2 gavages

spread 15 minutes apart. The volume of control diet given to mice was matched to the volume of ethanol diet consumed. Liver injury was determined by measuring serum ALT.

Western Blot Analysis. Total liver lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate). Heavy membrane (HM) enriched with mitochondria and cytosolic fractions were prepared as described previously (Ding, Ni et al. 2004). Briefly, liver tissues were homogenized in HIM buffer (200 mM mannitol, 70 mM sucrose, 5 mM Hepes, 0.5 mM EGTA (pH 7.5)) containing protease inhibitors using a dounce homogenizer. Homogenates were centrifuged at 1,000 x g to remove debris and nuclei, and the supernatant was centrifuged at 10,000 x g for 10 minutes to separate HM and cytosolic fractions. The supernatant was kept as the cytosolic fraction, and the pellet containing the HM fraction was further washed by centrifugation and re-suspended in HIM buffer. Protein (20-30 μg) was separated by a 12% SDS-PAGE gel before transfer to a PVDF membrane. Membranes were probed using appropriate primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Life Technologies, 34080).

RNA isolation and Real-Time qPCR. RNA was isolated from mouse livers using TRIzol reagent (Ambion, 15596-026) and was reverse-transcribed into cDNA using RevertAid Reverse Transcriptase (Fermentas, EP0442). qPCR was performed using SYBR Green chemistry (Biorad, 1725124). Primer sequences (5' - 3') for primers used in qPCR are: Accα F: CTCCAGGACAGCACAGATCA, R: TGACTGCCGAAACATCTCTG; Fasn F: TGGGTTCTAGCCAGCAGAGT, R: ACCACCAGAGACCGTTATGC; Srebp1 F: GATCAAAGAGGAGCCAGTGC, R: TAGATGGTGGCTGCTGAGTG; Acox1 F: CAGGAAGAGCAAGGAAGTGG, R: CCTTTCTGGCTGATCCCATA; Cpt1α F:

CCAGGCTACAGTGGGACATT, R: GAACTTGCCCATGTCCTTGT; Pparα F: ATGCCAGTACTGCCGTTTTC, R: GGCCTTGACCTTGTTCATGT; β-actin F: TGTTACCAACTGGGACGACA, R: GGGGTGTTGAAGGTCTCAAA. Real-time qPCR results were normalized to β-actin and expressed as fold over WT control (water binge or control diet, where appropriate).

Histology. For hematoxylin and eosin (H&E) staining, formalin-fixed liver sections were embedded in paraffin and cut into 5 μm sections before staining with H&E. For 4HNE staining, 5 μm paraffin sections were incubated with 4HNE antibody (Alpha Diagnostics, HNE11-S, 1:250) using a standard immunohistochemistry procedure. Briefly, tissue sections were incubated with primary antibody for 30 minutes after deparaffinization and antigen retrieval in citrate buffer. Sections were then washed and incubated with signal stain boost (Cell Signaling, 8114S) for 30 minutes and developed using DAB substrate (Vector, SK4105). Tissues were counterstained with hematoxylin. Positive areas were determined by Image J and calculated as a percentage of liver area. For Oil Red O staining, tissues were fixed in 4% paraformaldehyde overnight at 4°C and then transferred to 20% sucrose and stored at 4°C for approximately 24 hours before mounting in O.C.T. compound and freezing at -20°C. 6 μm sections were used for staining. Briefly, tissues were washed twice with PBS and then incubated with 60% isopropanol for 1 minute. Tissues were dried in a 37°C incubator for approximately 10 minutes before incubating with Oil Red O solution. Oil Red O solution was prepared by adding 0.35g Oil Red O (Sigma, 0625) to 100 mL of 100% isopropanol, which was further diluted 1.7 times in water and filtered immediately before use. Slides were incubated with Oil Red O solution for 15 minutes. The Oil Red O solution was aspirated from the slides, and 60% isopropanol was added to the slides for several minutes to remove any residual Oil Red O. Slides were then washed in PBS and stained

for 30 seconds with hematoxylin (Sigma, GHS132) followed by more washes in dH_2O. Slides were mounted with glycerol (5:1 in PBS).

Measurement of Liver Triglycerides. Approximately 20-50 mg of liver tissue was ground into powder in liquid nitrogen using a mortar and pestle. Triglycerides were extracted by incubating the powdered tissue in a chloroform-methanol mix (2:1) for 1 hour with vigorous shaking. 200 µL of water was added to the samples, and the samples were vortexed and centrifuged at 3000 x g for 5 minutes to separate out the lipid phase. The lipid phase was removed and dried, and the remaining pellet containing lipids was dissolved in a *tert*-butanol and Triton X-114-Methanol (2:1) mixture. Triglyceride levels were measured by colorimetric assay using a kit from Pointe Scientific (T7532-500).

Electron microscopy. Tissues were fixed with 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) followed by 1% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 1016CX electron microscope.

Mitochondrial Respiration. Complex I (glutamate/malate) mitochondrial respiration was measured by Oroboros. Mouse liver mitochondria were freshly isolated after acute-binge ethanol treatment and stored on ice until use. To isolate mitochondria, mouse liver tissue was minced in 10 mL of mitochondria isolation buffer (70 mM sucrose, 210 mM Mannitol, 5 mM HEPES, 1 mM EDTA) with 0.5% BSA using 4 strokes of a dounce homogenizer. The samples were then centrifuged at 800 x g for 10 minutes to remove debris and nuclei, and the supernatant was decanted through cheesecloth before centrifuging it at 8000 x g for 10 minutes. The pellet containing mitochondria was resuspended in 100 µL of mitochondria isolation buffer and washed by centrifugation at 8000 x g before final resuspension in 50 µL of mitochondria isolation buffer. Mitochondrial respiration was assessed using 500 µg of protein. We measured

State 3, or ADP-dependent, respiration by adding the substrates glutamate (5 μM) and malate (5 μM) along with a limiting amount of ADP (0.45 μM) to isolated liver mitochondria after acute-binge treatment. State 3 respiration represents respiration that is coupled to ATP synthesis because substrate is present along with ADP. We measured State 4, or ADP-independent, respiration by adding oligomycin (1 μM) to totally deplete ADP levels after measurement of State 3 respiration. State 4 respiration represents respiration that is not coupled to ATP synthesis because substrate is present, but ADP is not. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.1 μM) was then added to uncouple mitochondria in order to measure maximal respiratory capacity. Finally, the electron transport chain complex III and I inhibitors antimycin (2 μM) and rotenone (5 μM) were added to measure non-mitochondrial oxygen consumption of the sample. State 3 respiration was calculated using the peak plateau after ADP addition. State 4 respiration was calculated using the steady state value after oligomycin addition. Maximal respiratory capacity was calculated using the peak after FCCP addition. The steady state value after antimycin and rotenone addition was subtracted from all values, and results were normalized to protein concentration, which was confirmed after completion of the respiration assay. Final results were normalized to individual controls for WT and Parkin KO mice.

Cytochrome C Oxidase (COX) activity. COX activity was measured using mouse liver total protein lysates by a colorimetric assay from Sigma-Aldrich (CYTOCOX1) according to the manufacturer's instructions.

Statistical Analysis. Statistical analysis was conducted with a one-way ANOVA analysis followed by a Holm-Sidak test or by a Student's T-test as indicated. A $p<0.05$ was considered significant.

4.4 Results

4.4.1 Parkin KO Mice had Increased Liver Injury after Alcohol Administration.

WT and Parkin KO mice were treated with alcohol using two alcohol models: acute-binge and Gao-binge. Liver injury was determined by measuring serum ALT after alcohol treatment, and both WT and Parkin KO mice had increased liver injury after alcohol treatment compared to control treatments in both alcohol models. Parkin KO mice also had increased liver injury compared to WT mice after both acute-binge and Gao-binge alcohol treatments, but the difference in injury between WT and Parkin KO mice was only statistically significant in the Gao-binge model. Basal ALT levels were similar between WT and Parkin KO mice in both alcohol models (**Figure 4.4.1 A-B**). Liver injury was also overall higher in both WT and Parkin KO mice treated with alcohol using the Gao-binge model compared to mice treated with alcohol using the acute-binge model, as expected (Bertola, Park et al. 2013) (**Figure 4.4.1 A-B**). Alcohol consumption is well known to induce Cytochrome P450 2e1 (Cyp2e1) expression, which can further cause oxidative stress in the liver (Robin, Sauvage et al. 2005, Bansal, Liu et al. 2010, Leung and Nieto 2013). WT and Parkin KO mice both had marked inductions of Cyp2e1 after alcohol treatment with the Gao-binge model, but the changes of Cyp2e1 were mild with the acute-binge model. There were no differences in Cyp2e1 levels between WT and Parkin KO mouse livers, suggesting that increased liver injury in Parkin KO mice compared to WT mice was not due to elevated induction of Cyp2e1 (**Figure 4.4.1 C-D**).

Figure 4.4.1

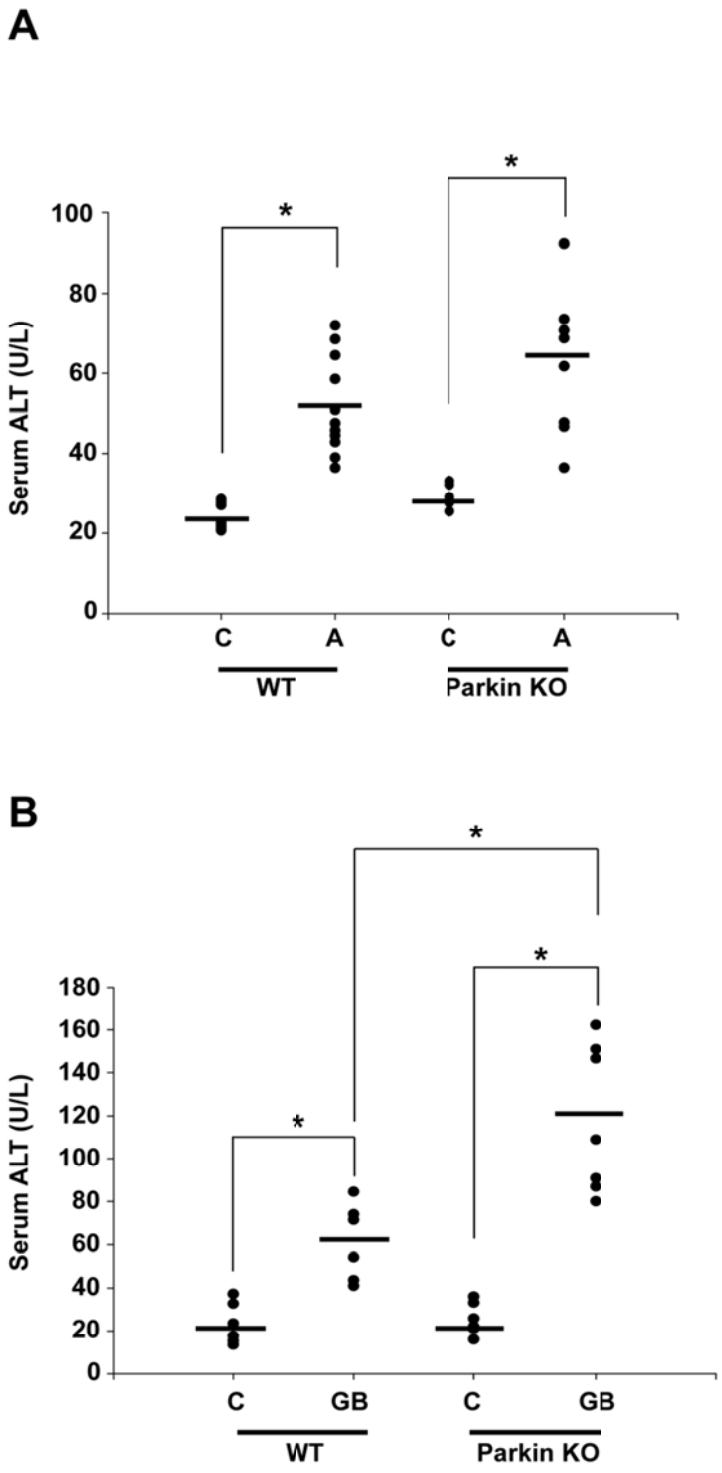

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Figure 4.4.1 Cont.

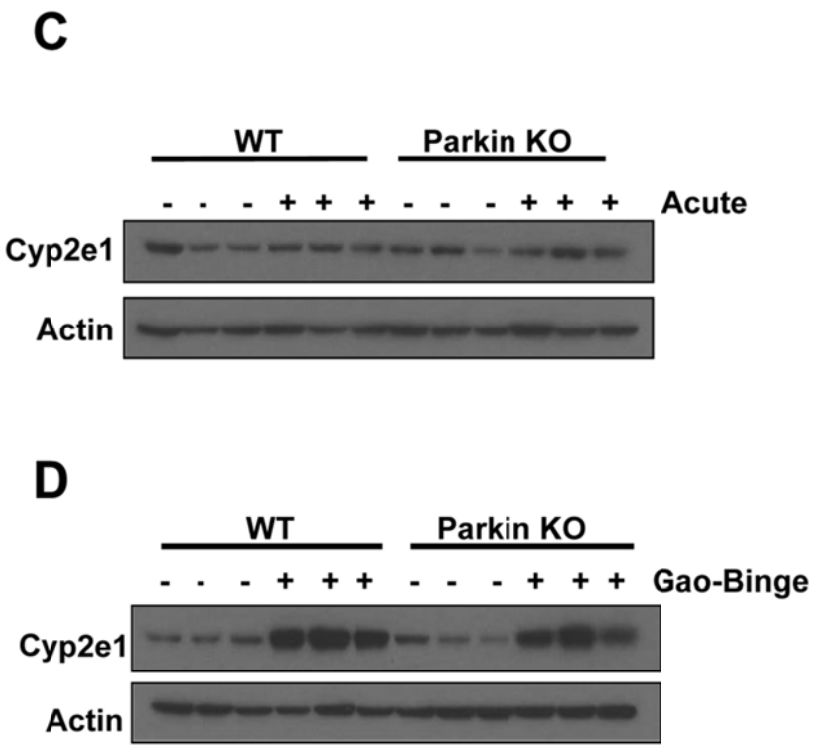
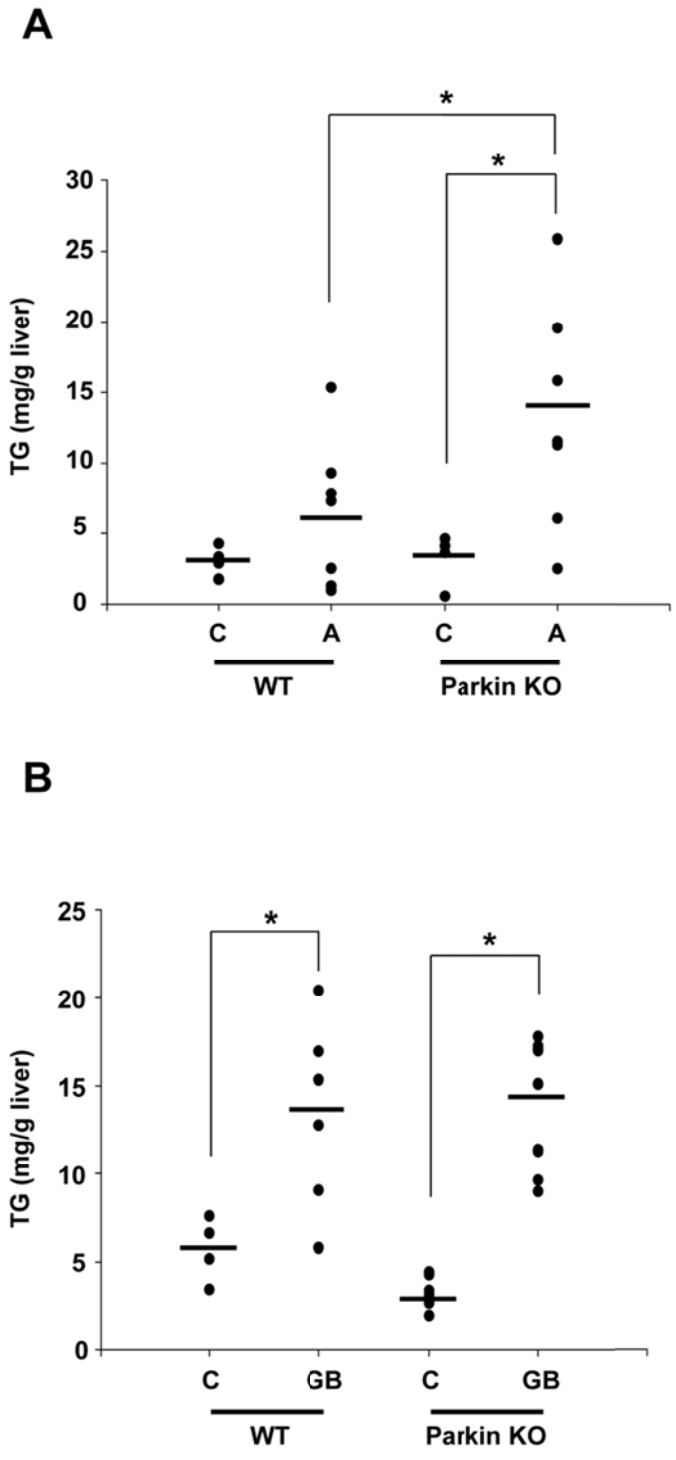


Figure 4.4.1: Alcohol induced more liver injury in Parkin KO mice. A-B: Serum ALT levels were measured from WT and Parkin KO mice after treatment with alcohol using the acute-binge (**A**) or Gao-binge (**B**) model. Data shown are means ± S.E. (n $\geq$ 5 mice per group. *p<0.05 by one-way ANOVA, A=acute-binge, GB=Gao-binge). **C-D:** WT and Parkin KO mice were treated with the acute-binge (**C**) or Gao-binge (**D**) model, and liver lysates were analyzed by western blot. β-actin was used as a loading control.

4.4.2 Parkin KO Mice had Increased Steatosis Compared to WT Mice after Acute-binge but not Gao-binge Treatment.

Treatment with alcohol using both the acute-binge and Gao-binge models caused liver steatosis in WT and Parkin KO mice, which was demonstrated by elevated liver triglycerides (**Figure 4.4.2 A-B**) and increased visualization of lipid droplets by H&E staining (**Figure 4.4.2 C-D**), Oil Red O staining (**Figure 4.4.2 E-F**), and EM (**Figure 4.4.2 G-J**). The Gao-binge model produced more macrosteatosis compared to the acute-binge model in both WT and Parkin KO alcohol-treated mouse livers (**Figure 4.4.2 C-F**). In addition, Parkin KO mice developed greater liver steatosis compared to WT mice after acute-binge alcohol treatment. Livers from acute-binge treated Parkin KO mice had significantly increased triglyceride levels compared to acute-binge treated WT mouse livers (**Figure 4.4.2 A**), which was confirmed by H&E and Oil Red O staining (**Figure 4.4.2 C, 4.4.2 E**)**.** Acute-binge treated Parkin KO mouse livers also had a significant increase in the number of lipid droplets per cell compared to acute-binge treated WT mouse livers (**Figure 4.4.2 G-H**). However, the Gao-binge model caused greater triglyceride accumulation in WT mouse livers compared to the acute-binge model, but triglyceride levels were similar between Parkin KO Gao-binge and acute-binge treated mouse livers (**Figure 4.4.2 A-B**). Overall, these data suggest that acute-binge caused greater liver fat accumulation in Parkin KO mice compared to WT mice. In addition, the Gao-binge model caused greater steatosis in WT mouse livers compared to WT mouse livers treated with the acute-binge model, but the Gao-binge and acute-binge models both caused similar levels of steatosis in Parkin KO mouse livers.

Figure 4.4.2


A
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TG (mg/g liver)
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WT
Parkin KO
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TG (mg/g liver)
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Parkin KO

Figure 4.4.2 Cont.

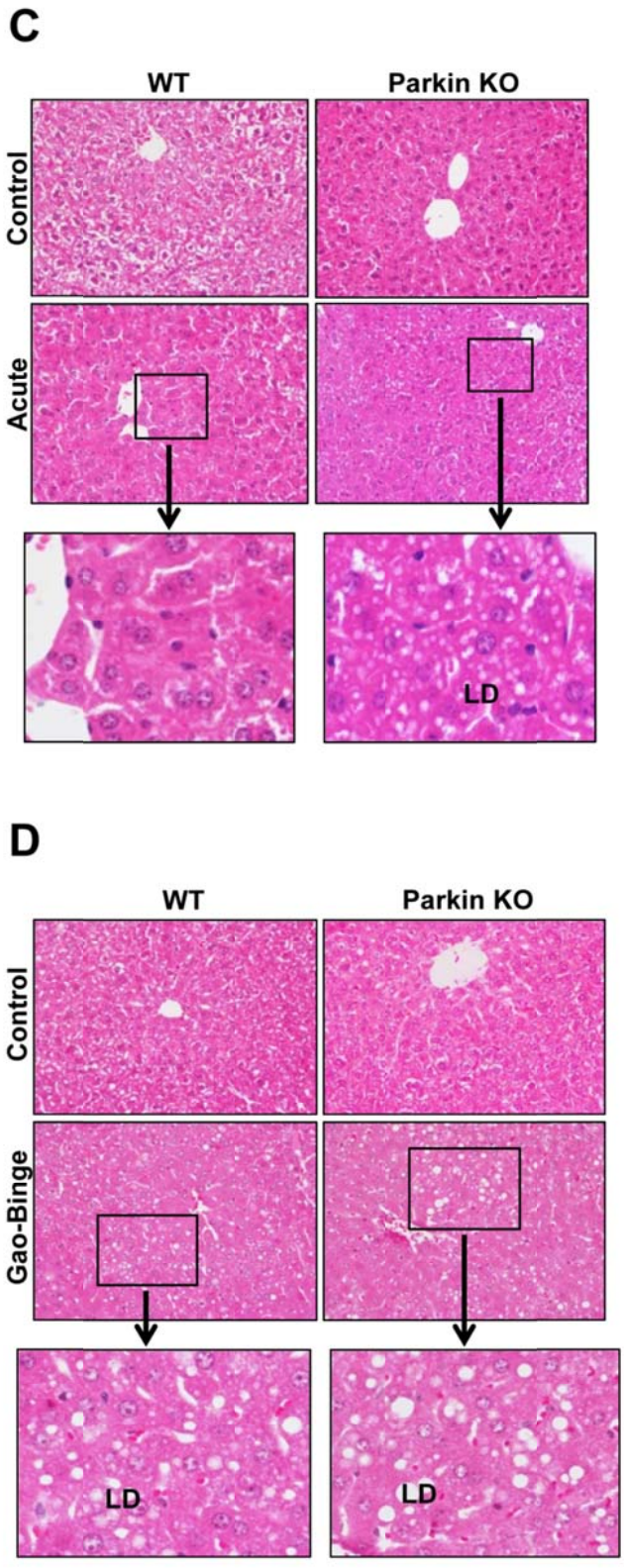


Figure 4.4.2 Cont.

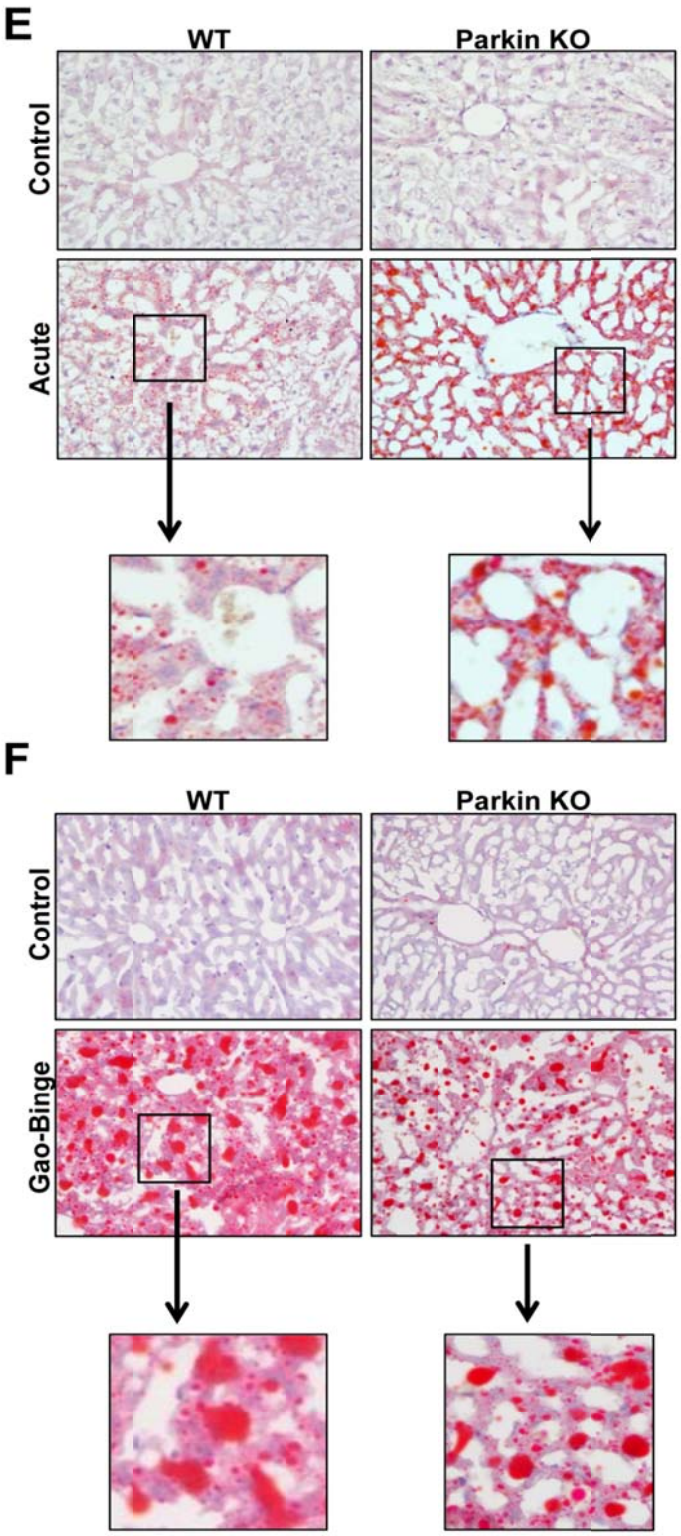
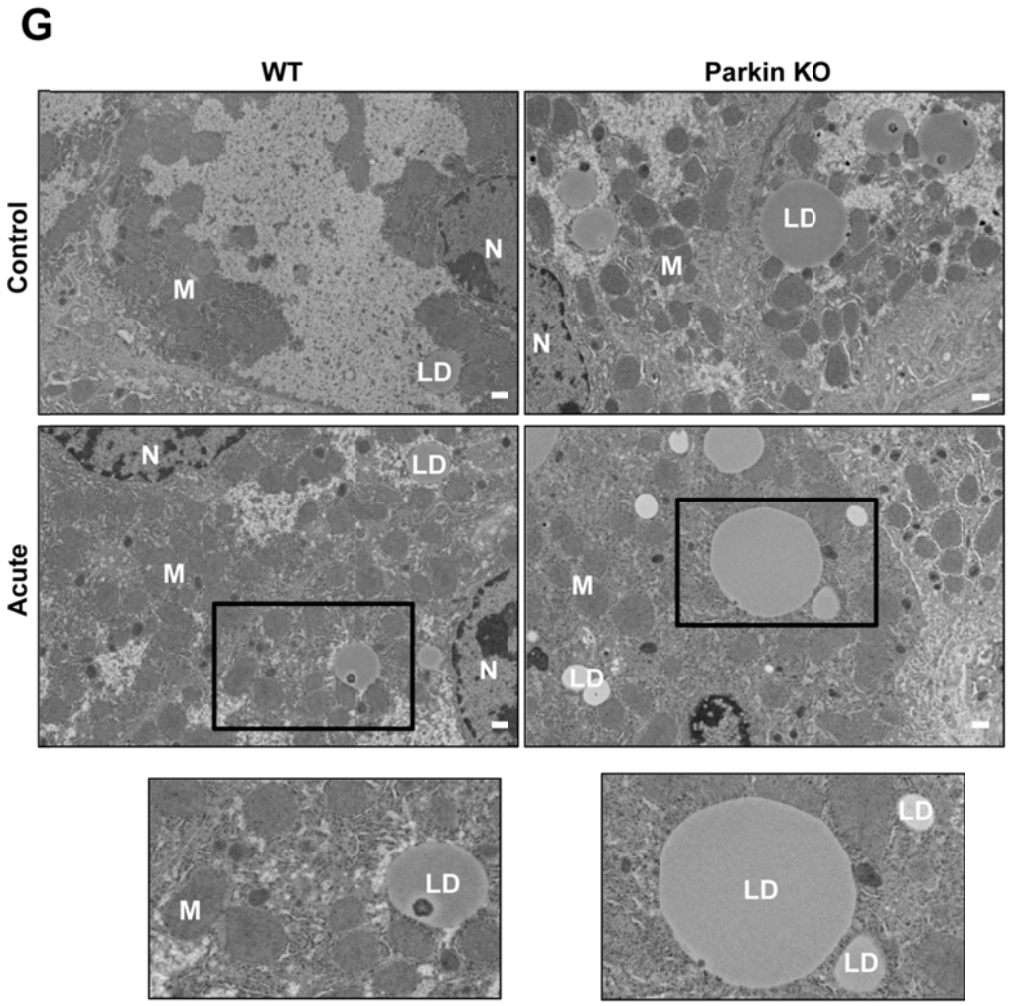


Figure 4.4.2 Cont.



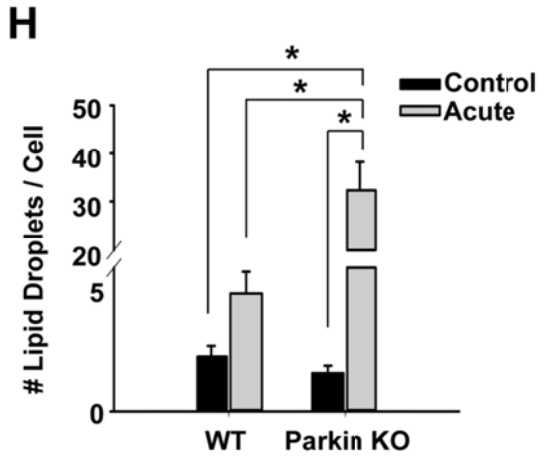
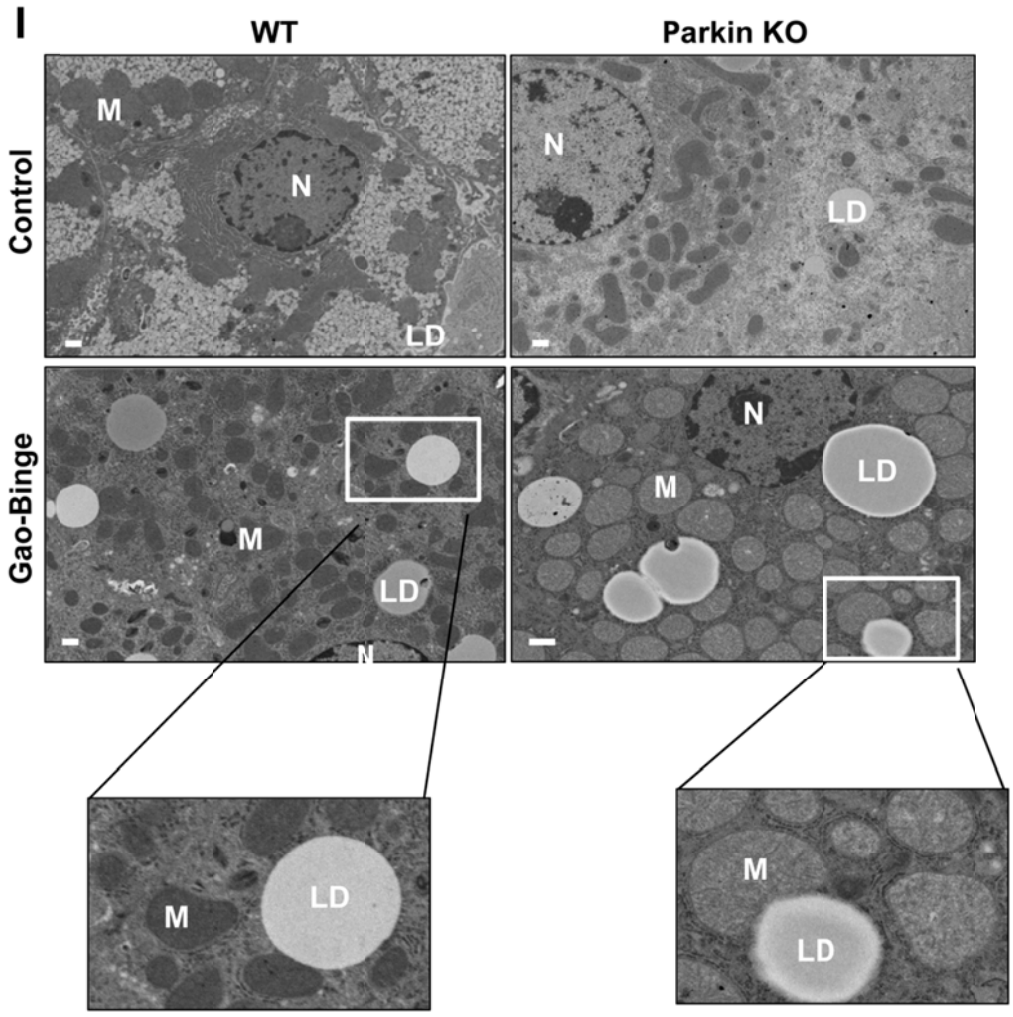


Figure 4.4.2 Cont.



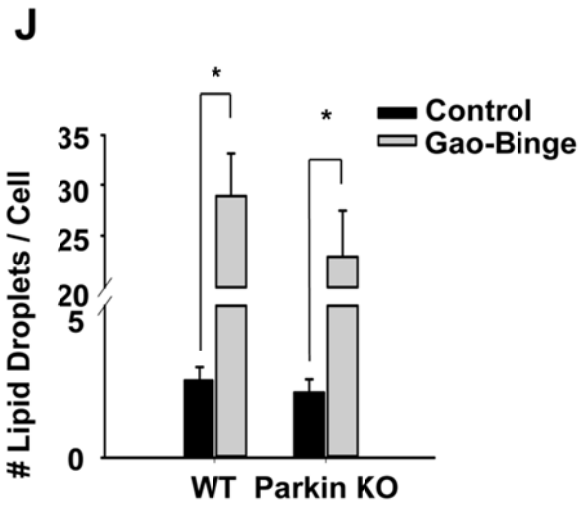


Figure 4.4.2 Cont.

Figure 4.2.2: Parkin KO mice had increased liver steatosis compared to WT mice after acute-binge, but not Gao-binge, treatment. A-B: Liver triglycerides (TG) were measured for WT and Parkin KO mice after acute-binge (**A**) and Gao-binge (**B**) alcohol treatment. Data shown are means ± S.E. (n= 4 for controls and ≥ 6 for alcohol treated mice. *p<0.05 by one-way ANOVA, A=acute-binge, GB=Gao-binge). **C-D:** Representative H&E images from the acute-binge model (**C**) and the Gao-binge model (**D**) are shown with boxed areas enlarged. (LD=lipid droplet, 200x magnification). **E-F:** Representative images are shown for Oil Red O staining for the acute-binge model (**E**) and for the Gao-binge model (**F**) with boxed areas enlarged (200x magnification). **G:** Representative EM images are shown for acute-binge treated mice with boxed areas enlarged (Bar=500 nm, N=nucleus, M=mitochondria, LD=lipid droplet). **H:** Quantification of lipid droplets per cell in acute-binge treated mice. Data shown are means ± S.E. (n= ≥ 10 images per mouse from 2 mice per group. *p<0.05 by one-way ANOVA). **I:** Representative EM images are shown for Gao-binge treated mice with boxed areas enlarged (Bar=500 nm, N=nucleus, M=mitochondria, LD=lipid droplet). **J:** Quantification of lipid droplets per cell in Gao-binge treated mice. Data shown are means ± S.E. (n= ≥ 10 images per mouse from 2 mice per group. *p<0.05 by one-way ANOVA).

4.4.3 Steatosis in WT and Parkin KO Mouse Alcohol-treated Livers was not due to Fatty Acid Synthesis.

Alcohol has been shown to cause steatosis in the liver by elevating fatty acid synthesis in the intragastric infusion (Ji, Chan et al. 2006) and chronic alcohol feeding models (You, Fischer et al. 2002). To determine if increased liver steatosis after alcohol treatment in WT and Parkin KO mouse livers was due to increased fatty acid synthesis, expression levels of several fatty acid synthesis genes were measured in mouse livers after alcohol treatment using both the acute and Gao-binge models. Gene expression levels of the fatty acid synthesis enzymes acetyl-coA carboxylase-alpha (*Accα*) and fatty acid synthase (*Fasn*) did not significantly change after alcohol treatment by either the acute-binge or Gao-binge alcohol model in WT or Parkin KO mice compared to their individual controls. However, basal expression levels of these genes were decreased in Parkin KO mouse livers compared to WT mice in the acute model (**Figure 4.4.3 A-B**). Liver gene expression levels for sterol regulatory element-binding transcription factor 1 (*Srebp1*) were significantly decreased in WT mouse ethanol-treated livers from both the acute and Gao-binge models compared to control livers. *Srebp1* gene expression was overall decreased in Parkin KO mouse livers compared to WT livers but was not significantly affected in Parkin KO mouse livers after alcohol treatment compared to Parkin KO control livers for either alcohol model (**Figure 4.4.3 A-B**). Protein levels for Accα and Fasn were similar to mRNA levels (**Figure 4.4.3 C-D**). These data suggest that alcohol-induced steatosis in WT and Parkin KO mouse livers was not likely due to an increase in fatty acid synthesis.

Figure 4.4.3

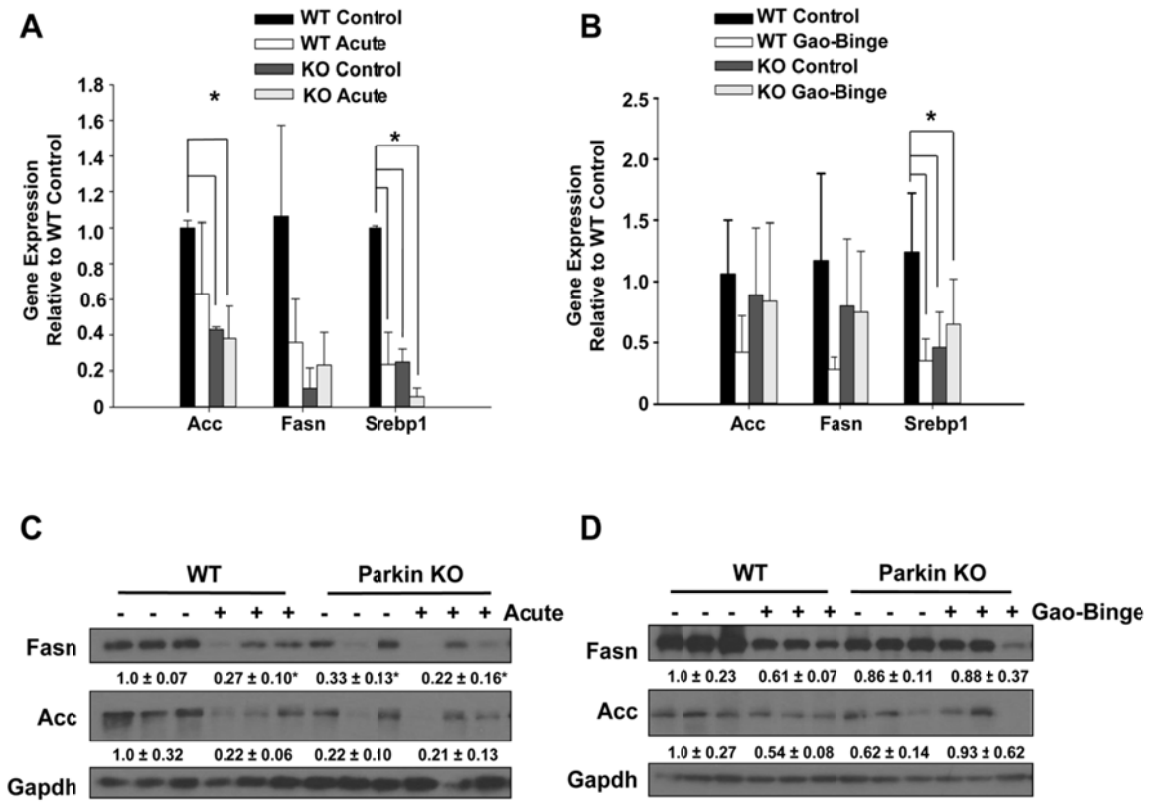


Figure 4.4.3: Steatosis in WT and Parkin KO alcohol-treated livers was not due to fatty acid synthesis. A-B: WT and Parkin KO mice were treated with the acute-binge (**A**) or Gao-binge (**B**) model, and RNA from mouse livers was used to measure gene expression by qPCR. Results were normalized to β-actin and expressed as fold change compared to WT control. Data shown are means ± S.D. (n = 3-4 mice per group. *p<0.05 by one-way ANOVA). **C-D:** WT and Parkin KO mice were treated with the acute-binge (**C**) or Gao-binge (**D**) model, and protein from mouse livers was used to measure protein expression by Western blot. Results were normalized to Gapdh, and densitometry results are expressed as fold change compared to WT control. Data shown are means ± S.E. (n = 3 mice per group. *p<0.05 by one-way ANOVA).

4.4.4. Greater Steatosis in Parkin KO Mouse Livers after Acute-binge Alcohol Treatment Compared to WT Mouse Livers was due to Decreased β-oxidation.

Alcohol-induced inhibition of β-oxidation has been shown to cause fatty liver (Sozio and Crabb 2008). To determine if increased steatosis in WT and Parkin KO alcohol-treated mouse livers was due to decreased fat degradation by β-oxidation, expression levels of several genes involved in the β-oxidation pathway were measured including the enzymes peroxisomal acyl-coenzyme A oxidase 1 (*Acox1*) and carnitine palmitoyltransferase 1 alpha (*Cpt1α*) and the transcription factor peroxisome proliferator-activated receptor alpha (*Pparα*). In the acute-binge model, gene expression levels for *Acox1* and *Pparα* were significantly increased after alcohol treatment in WT mouse livers compared to controls. Expression of *Cpt1α* was also increased in acute-binge treated WT mouse livers compared to controls, but this increase was not statistically significant. However, expression levels of these genes in Parkin KO acute-binge treated mouse livers did not increase compared to Parkin KO control treated livers. Furthermore, the expression levels of *Acox1* and *Ppara* were significantly decreased in Parkin KO acute-binge treated mouse livers compared to WT mouse livers. The expression level of *Cpt1α* was also decreased in acute-binge treated Parkin KO mouse livers compared to acute-binge treated WT mouse livers, but this decrease was not statistically significant. There was no difference in basal expression levels for these genes between WT and Parkin KO mice (**Figure 4.4.4. A**). These data suggest that liver β-oxidation was likely activated as a protective mechanism in WT mouse livers after acute-binge alcohol treatment to help decrease liver steatosis. However, β-oxidation genes were not induced after acute-binge treatment in Parkin KO mouse livers, suggesting that liver β-oxidation may have been inhibited in Parkin KO mice treated with acute-binge. Decreased or inhibited β-oxidation in Parkin KO acute-binge treated mouse livers may likely explain the greater levels of steatosis seen in Parkin KO acute-binge treated mouse livers compared to WT acute-binge treated livers (**Figure 4.4.2 A, C, E, G, H**).

Even though expression of β-oxidation genes was increased in WT mouse livers after acute ethanol treatment, expression of these genes was not induced by Gao-binge alcohol treatment in either WT or Parkin KO mouse livers compared to controls, suggesting that β-oxidation may have been inhibited in both WT and Parkin KO mouse livers after Gao-binge alcohol treatment. In fact, expression of β-oxidation genes was actually decreased in both WT and Parkin KO mouse livers after Gao-binge alcohol treatment compared to control-diet fed mouse livers, but this decrease was only significant for *Cpt1a* gene expression in Parkin KO Gao-binge treated livers. Surprisingly, *Cpt1a* expression in Parkin KO mouse control-treated livers was significantly increased compared to WT control livers, suggesting that Parkin KO mice had higher levels of β-oxidation when fed the control diet compared to WT mice. There were no significant differences between Gao-binge treated WT and Parkin KO mouse livers for any of the measured β-oxidation genes (**Figure 4.4.4 B**). Together, these data suggest that β-oxidation was increased in acute-binge treated WT mouse livers, which likely acted as an adaptive mechanism to reduce alcohol-induced liver steatosis. However, this protective adaptive mechanism seemed to be down-regulated in Parkin KO acute-binge treated mouse livers, which could be a reason for their increased alcohol-induced liver steatosis compared to WT mice. The protective response of β-oxidation seemed to be inhibited in both WT and Parkin KO mouse livers after Gao-binge alcohol treatment, which may explain why the levels of steatosis in WT and Parkin KO mouse livers were increased to similar levels after alcohol treatment using the Gao-binge model.

Figure 4.4.4

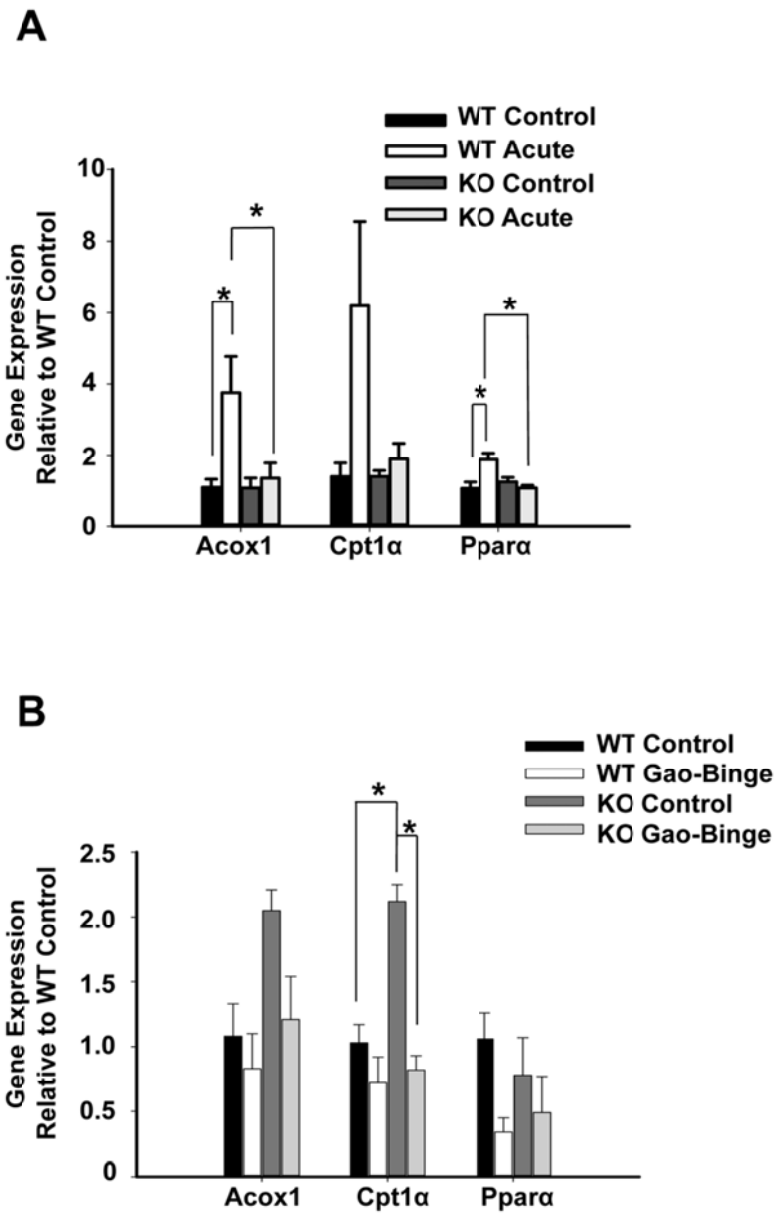


Figure 4.4.4: Greater steatosis in WT and Parkin KO alcohol-treated livers was due to decreased β-oxidation. A-B: WT and Parkin KO mice were treated with the acute-binge (**A**) or Gao-binge (**B**) model, and RNA from mouse livers was used to measure gene expression by qPCR. Results were normalized to β-actin and expressed as fold change compared to WT control. Data shown are means ± S.D. (n = 3-4 mice per group. *p<0.05 by one-way ANOVA).

4.4.5 Reduced Mitophagy in Parkin KO Mice after Alcohol Treatment.

Because decreased mitophagy could potentially lead to an increased population of damaged and dysfunctional mitochondria and subsequent liver injury, we compared levels of mitophagy between WT and Parkin KO mice after alcohol treatment using both the acute-binge and Gao-binge models. First, we determined if Parkin translocated to mitochondria in WT mouse livers after alcohol treatment. Parkin did not translocate to mitochondria after acute-binge alcohol treatment for 16 hours (**Figure 4.4.5 A**). However, Parkin did translocate to mitochondria after Gao-binge alcohol treatment in WT mouse livers (**Figure 4.4.5 B**), suggesting that Parkin-induced mitophagy may occur in WT mouse livers after Gao-binge alcohol treatment.

To further determine mitophagy levels between WT and Parkin KO mouse livers, we measured protein expression of the autophagy adaptor protein p62 and the autophagosome membrane protein microtubule-associated protein light chain 3 (LC3) in total liver lysates from WT and Parkin KO mice after acute-binge and Gao-binge treatment. There were no significant differences in p62 or LC3-II protein expression after alcohol treatment compared to controls in either alcohol model for WT or Parkin KO mice. In addition, there were no differences in expression of these proteins between WT and Parkin KO control mice (**Figure 4.4.5 C-F**).There was also no degradation of the mitochondrial outer membrane proteins Translocase of outer membrane 20 (Tom20) or Voltage-dependent anion channel (VDAC) or of the mitochondrial matrix protein Heat shock protein 60 (Hsp60) after alcohol treatment by either the acute-binge or Gao-binge model in WT or Parkin KO mice (**Figure 4.4.5 C-F**). These data suggest that mitophagy levels may be too mild to detect differences between WT and Parkin KO mice by Western blot analysis. Alternatively, mice may adapt to alcohol-induced mitochondrial damage by compensatory mitochondrial biogenesis, which can further offset mitophagic degradation determined by Western blot analysis.

We next investigated if there were differences in the number of autophagosomes (Avi) and autolysosomes (Avd) containing mitochondria (hereafter referred to as mitophagosomes) between WT and Parkin KO mouse livers after alcohol treatment using electron microscopy (EM). In the acute-binge and Gao-binge alcohol models, the levels of mitophagy were significantly increased after alcohol treatment in WT mouse livers. However, the number of mitophagosomes was significantly decreased in Parkin KO mice compared to WT mice after alcohol treatment (**Figure 4.4.5 G, I, J, L**). The total number of autophagic vacuoles after alcohol treatment was also slightly decreased in Parkin KO mice compared to WT mice, but these data were not statistically significant (**Figure 4.4.5 G, H, J, K**). These data suggest that alcohol-induced mitophagy was decreased in Parkin KO mouse livers compared to WT mouse livers.

Figure 4.4.5

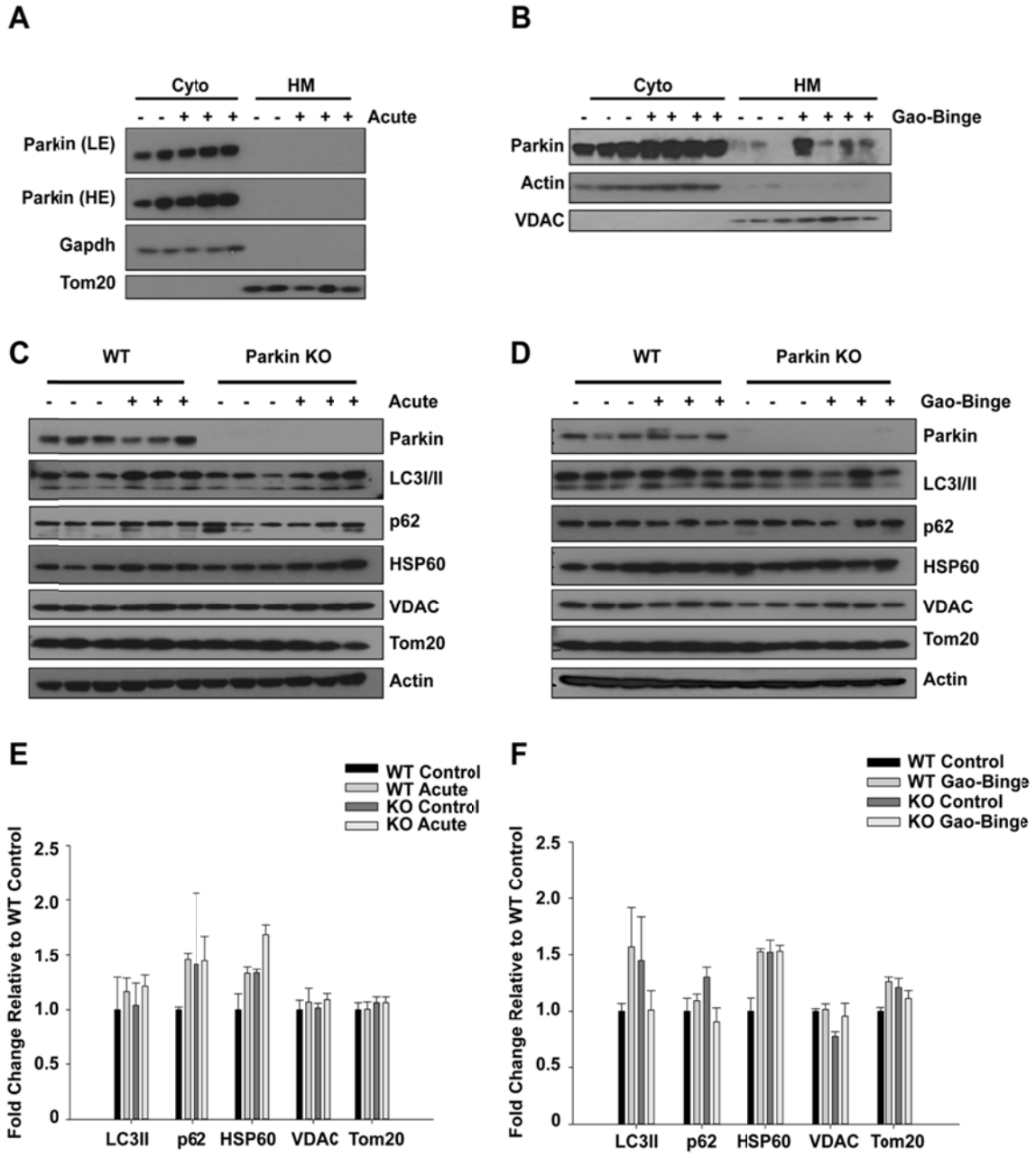

A
Cyto
HM
Acute
Parkin (LE)
Parkin (HE)
Gapdh
Tom20
B
Cyto
HM
Gao-Binge
Parkin
Actin
VDAC
C
WT
Parkin KO
Acute
Parkin
LC3I/II
p62
HSP60
VDAC
Tom20
Actin
D
WT
Parkin KO
Gao-Binge
Parkin
LC3I/II
p62
HSP60
VDAC
Tom20
Actin
E
WT Control
WT Acute
KO Control
KO Acute
Fold Change Relative to WT Control
LC3II
p62
HSP60
VDAC
Tom20
F
WT Control
WT Gao-Binge
KO Control
KO Gao-Binge
Fold Change Relative to WT Control
LC3II
p62
HSP60
VDAC
Tom20

Figure 4.4.5 Cont.

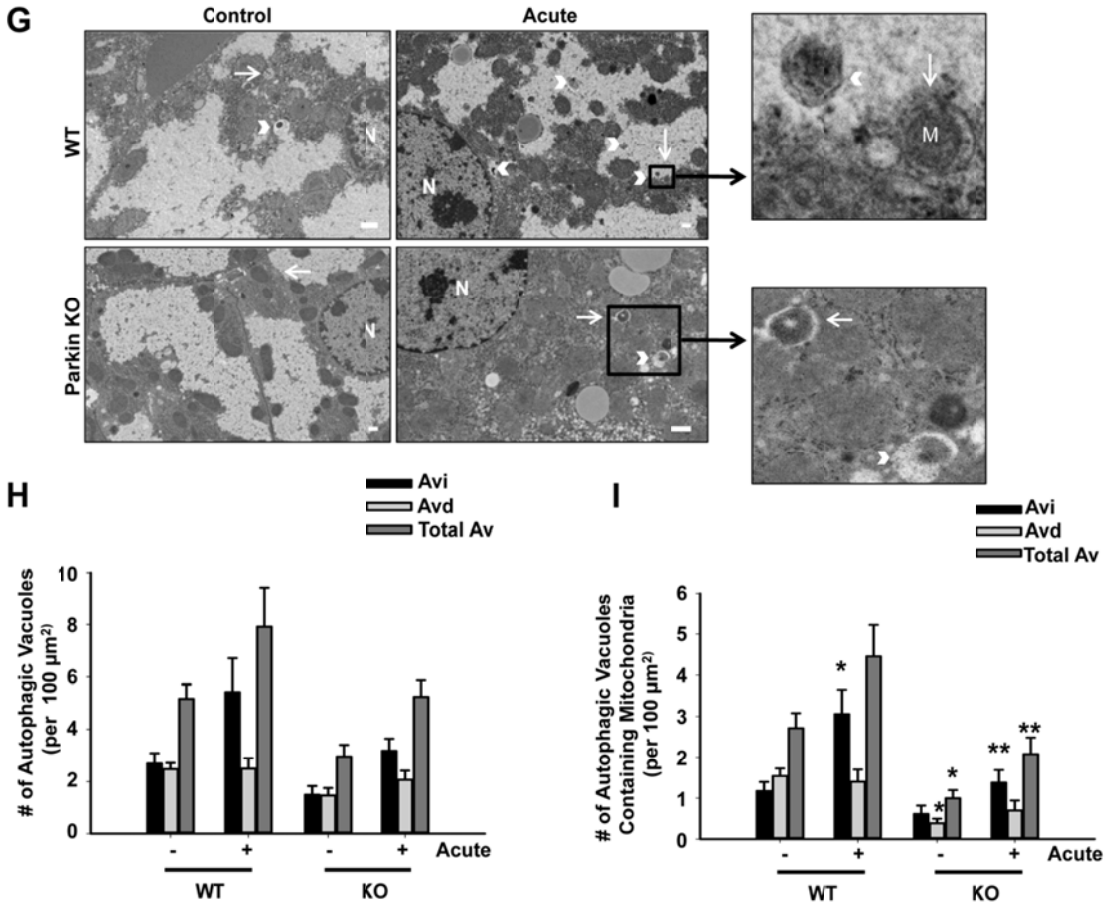


Figure 4.4.5 Cont.

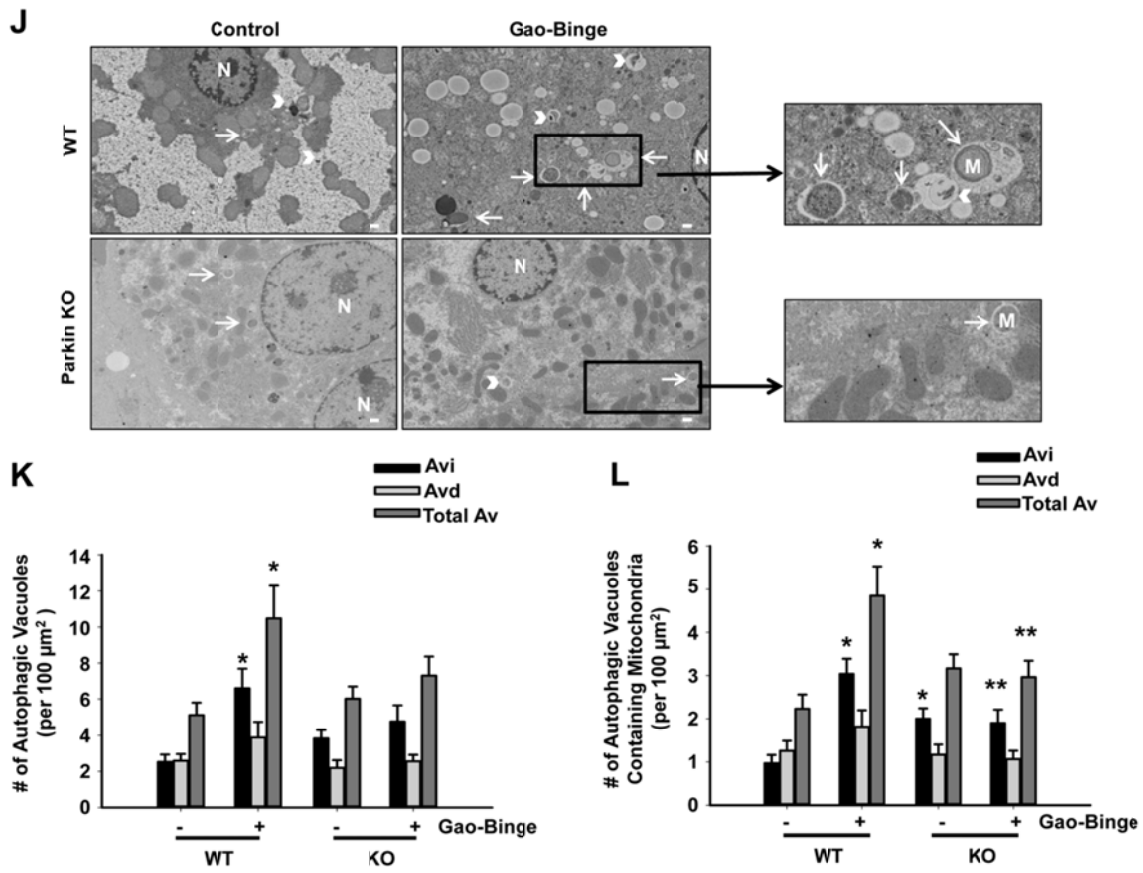
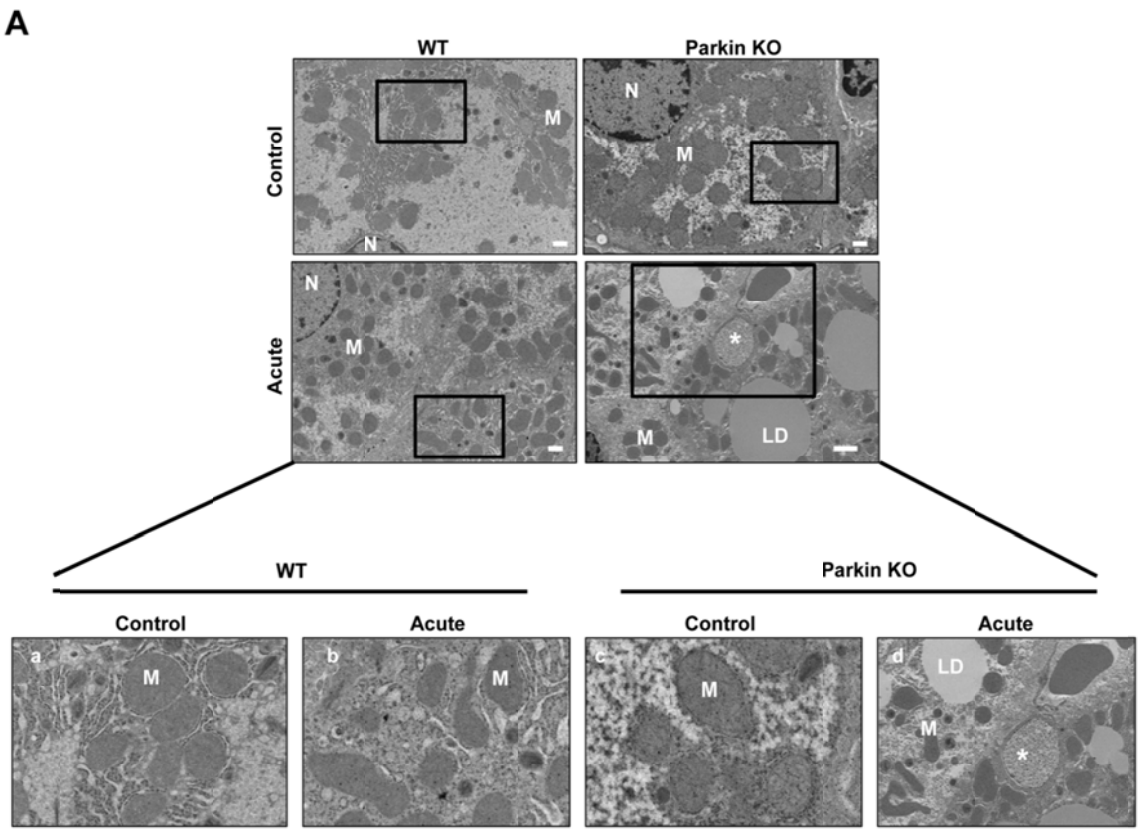


Figure 4.4.5: Decreased mitophagy in Parkin KO mice. A-B: WT and Parkin KO mice were treated with alcohol using the acute-binge (**A**) and Gao-binge (**B**) models, and liver cytosolic and HM fractions were isolated and analyzed by Western blot. β-actin and VDAC were used as loading controls. **C-D:** WT and Parkin KO mice were treated with alcohol using the acute-binge (**C**) or Gao-binge (**D**) model and liver lysates were used for Western blot analysis. β-Actin was used as a loading control. **E-F:** Densitometry quantification for blots in **C** and **D**, respectively**.** Data shown are means ± S. E. (n=3 mice per group. No significant differences among groups). **G:** Representative EM images are shown for the acute-binge model with boxed areas enlarged (arrows represent autophagosomes, and arrowheads represent autolysosomes. M=mitochondria, N=nucleus. Bar=500 nm). **H-I:** Quantification of the number of autophagic vacuoles (**H**) and mitophagosomes (**I**) from EM in **G**. Data shown are means ± S.E. (n= 1 mouse per group, at least 15 images quantified per mouse. *p<0.05 compared to WT control, **p<0.05 compared to WT alcohol by one-way ANOVA). **J:** Representative EM images are shown for the Gao-binge model with boxed areas enlarged (arrows represent autophagosomes, and arrowheads represent autolysosomes. M=mitochondria, N=nucleus. Bar=500 nm). **K-L:** Quantification of the number of autophagic vacuoles (**K**) and mitophagosomes (**L**) from EM in **J.** Data shown are means ± S.E. (n= 1 mouse per group, at least 15 images quantified per mouse. *p<0.05 compared to WT control, **p<0.05 compared to WT alcohol by one-way ANOVA).

4.4.6 Mitochondrial Morphological Changes in the Gao-binge Alcohol Model

Chronic alcohol feeding has been shown to cause changes to mitochondrial morphology (Han, Ybanez et al. 2012). Therefore, we determined if alcohol treatment using the acute-binge or Gao-binge alcohol models caused changes to mitochondrial morphology in WT and Parkin KO mouse livers by EM. Alcohol treatment by the acute-binge model did not cause any significant changes in mitochondrial morphology in WT mice compared to controls. However, some mitochondria in Parkin KO mouse livers appeared to have swelling after acute-binge treatment compared to control treated liver mitochondria, but most mitochondria did not appear to be significantly damaged by alcohol treatment (**Figure 4.4.6 A**). Alcohol treatment using the Gao-binge model caused significantly damaged and swollen mitochondria in Parkin KO mouse livers where approximately 1% of mitochondria were so severely swollen and damaged that they lacked cristae. Only approximately 0.14% of mitochondria were this severely damaged in Parkin KO mice treated with the acute-binge model (**Figure 4.4.6 B-D**). These severely swollen and damaged mitochondria were not seen in WT mice after alcohol treatment with either model. In addition, Parkin KO mice had some liver mitochondria that were elongated after Gao-binge alcohol treatment, but WT mice appeared to have more elongated mitochondria after alcohol treatment by Gao-binge while Parkin KO mouse livers had more swollen mitochondria (**Figure 4.4.6 F)**. Elongation of mitochondria is thought to be a cellular adaptive mechanism to chronic alcohol treatment (Han, Ybanez et al. 2012). Acute-binge did not have a significant effect on mitochondria elongation in either WT or Parkin KO mice (**Figure 4.4.6 E**). Overall, these results suggest that alcohol produced more damaged mitochondria in Parkin KO mouse livers compared to WT mouse livers, which may be due to an inability for Parkin KO mouse liver mitochondria to adapt to alcohol treatment or due to Parkin KO mouse livers having decreased mitophagy.

Figure 4.4.6


A
WT
Parkin KO
Control
Acute
N
M
LD
*
WT
Parkin KO
Control
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Control
Acute
a
b
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Figure 4.4.6 Cont.

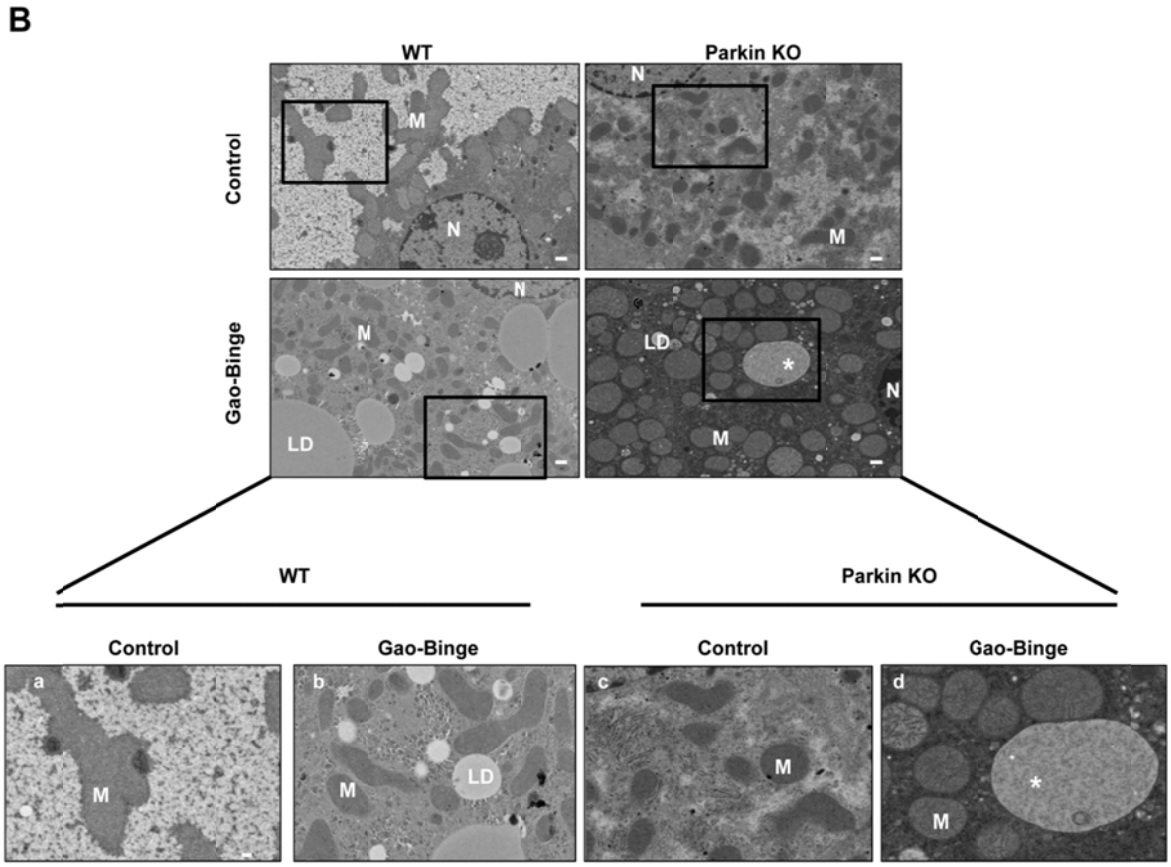

Figure 4.4.6 Cont.

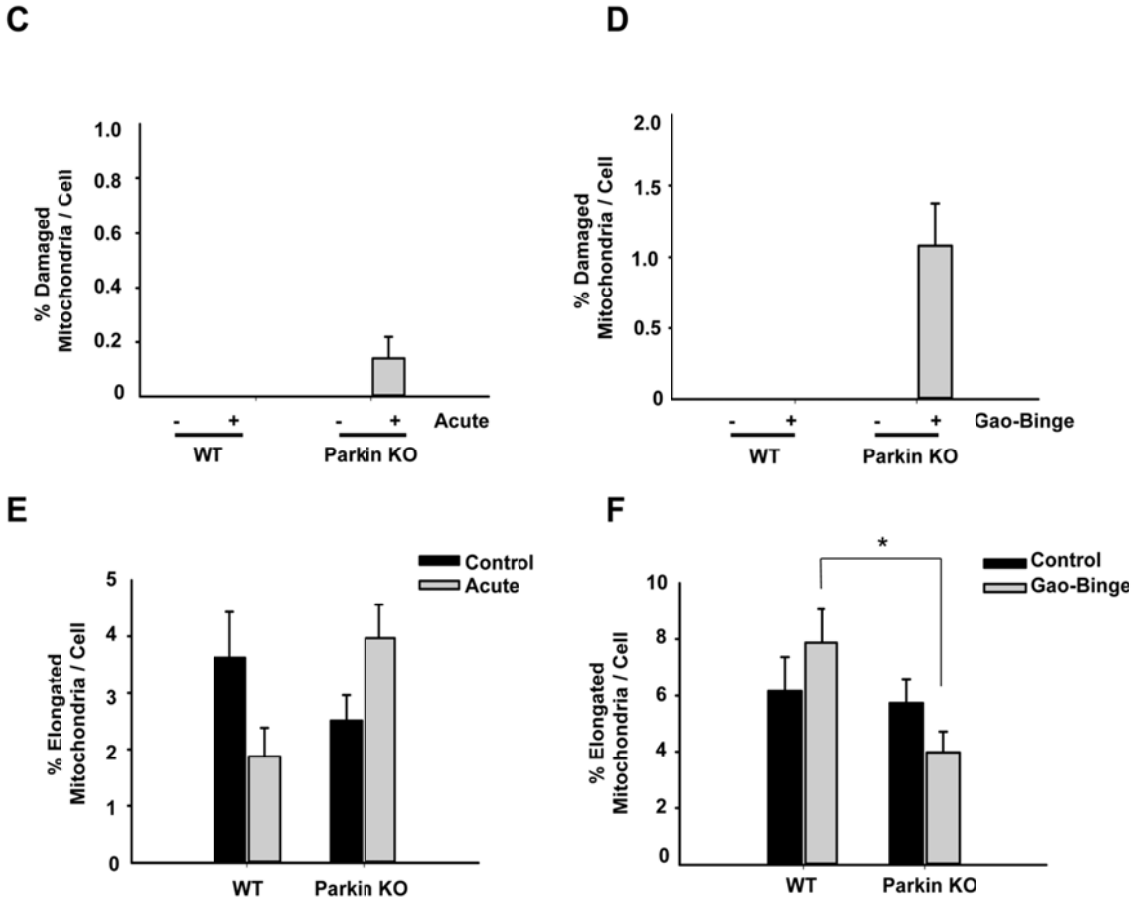

Figure 4.4.6: Analysis of mitochondrial morphology after alcohol treatment in WT and Parkin KO mice. **A-B:** Representative EM images are shown from WT and Parkin KO mice treated with the acute binge (**A**) or Gao-binge (**B**) model with boxed areas enlarged (a: WT control, b: WT alcohol, c: Parkin KO control, d: Parkin KO alcohol). N=nucleus. M=mitochondria. LD=lipid droplet. *=severely damaged mitochondria. Bar = 500 nm with the exception of figure **A(d)**, which is 2 μm. **C-D:** Quantification of the number of severely damaged mitochondria shown in figures **A(d)** and **B(d)** in WT and Parkin KO mice after acute-binge (**C**) or Gao-binge (**D**) (n= ≥ 10 images per mouse from 2 mice per group). **E-F:** Quantification of the number of elongated mitochondria per cell in WT and Parkin KO mice after acute-binge (**E**) or Gao-binge (**F**). Data shown are means ± S.E. (n= ≥ 10 images per mouse from 2 mice per group, * $p<0.05$ by one-way ANOVA).

4.4.7 Mitochondrial Respiration and COX Activity were Decreased in Parkin KO Mice after Alcohol Treatment.

To further determine if Parkin KO mouse liver mitochondria were more damaged than WT mouse liver mitochondria after alcohol treatment, we investigated if there were any differences in mitochondrial function between WT and Parkin KO mouse livers by measuring respiration rate and COX activity. We found that State 3 and State 4 respiration rates were both increased in WT mouse liver mitochondria after acute-binge treatment compared to controls, but this increase was not statistically significant (**Figure 4.4.7 A-B**). However, State 3 and State 4 respiration rates were decreased in Parkin KO mouse liver mitochondria after acute-binge treatment, which was statistically significant for State 3 respiration rates compared to acute-binge treated WT mice (**Figure 4.4.7 A-B**). Maximal respiratory capacity was also increased in WT mouse liver mitochondria after acute-binge treatment while it was decreased in Parkin KO mouse liver mitochondria (**Figure 4.4.7 C**). Basal respiration rates were similar between WT and Parkin KO mouse liver mitochondria (**Figure 4.4.7 A-C**). COX activity was slightly decreased in WT mouse livers after acute-binge treatment, but this decrease was not statistically significant. However, COX activity was significantly decreased in Parkin KO mouse livers after acute-binge treatment compared to WT control, suggesting that Parkin KO mouse liver mitochondria were more damaged after acute-binge treatment than WT mouse liver mitochondria. Parkin KO mouse livers also had slightly decreased basal levels of COX activity compared to WT mouse livers, but this difference was not significant (**Figure 4.4.7 D**). Interestingly, there were no significant differences in COX activity between control diet and Gao-binge treated WT or Parkin KO mice, but Parkin KO mice had slightly less COX activity than WT mice after Gao-binge treatment (**Figure 4.4.7 E**). These data suggest that liver mitochondria in WT mice were likely adapting to alcohol treatment, and Parkin KO mouse mitochondria were unable to adapt as well as WT mice to alcohol treatment. Overall, these data suggest that Parkin KO mouse livers had

more mitochondrial damage initiated by alcohol treatment than WT mouse livers, which likely led to their increased liver injury in the acute-binge and Gao-binge models and increased steatosis in the acute-binge model compared to WT mice.

Figure 4.4.7

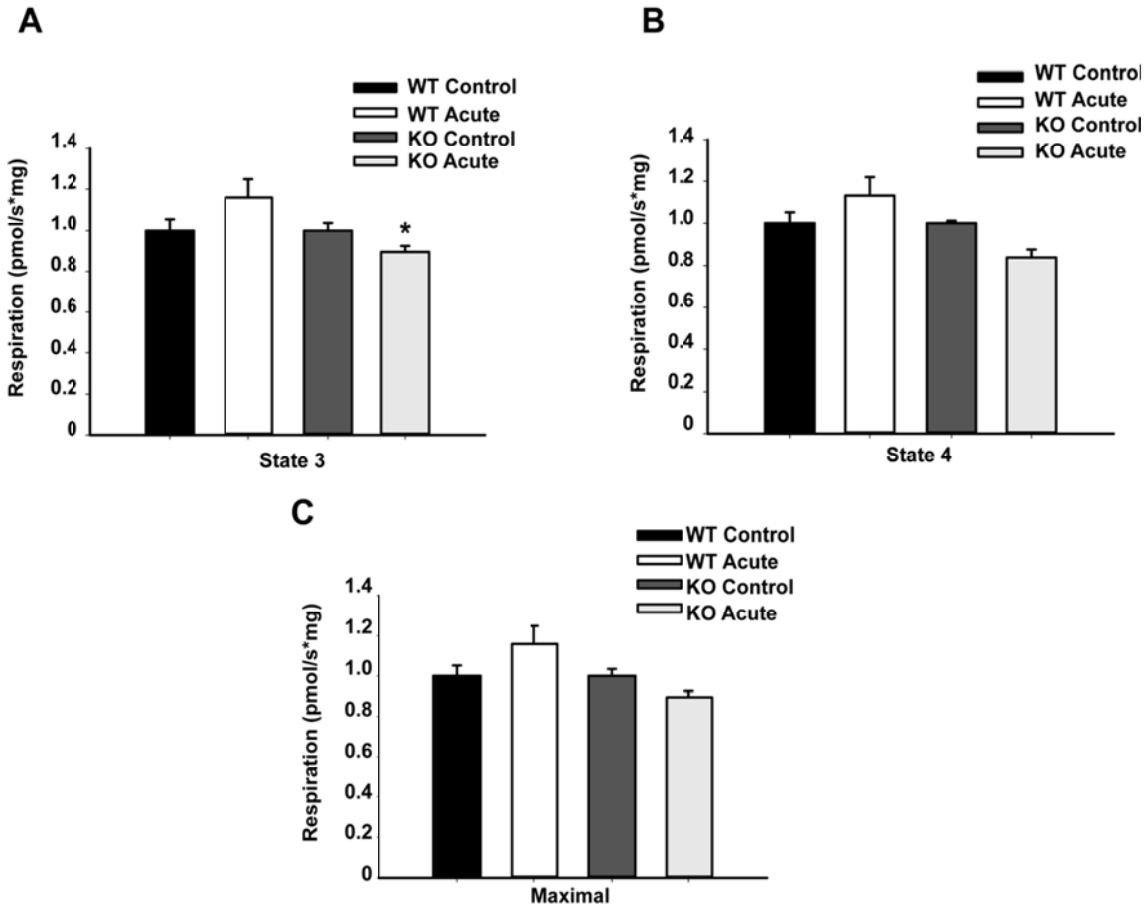
A
WT Control
WT Acute
KO Control
KO Acute
Respiration (pmol/s*mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
State 3
B
WT Control
WT Acute
KO Control
KO Acute
Respiration (pmol/s*mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
State 4
C
WT Control
WT Acute
KO Control
KO Acute
Respiration (pmol/s*mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Maximal

Figure 4.4.7 Cont.

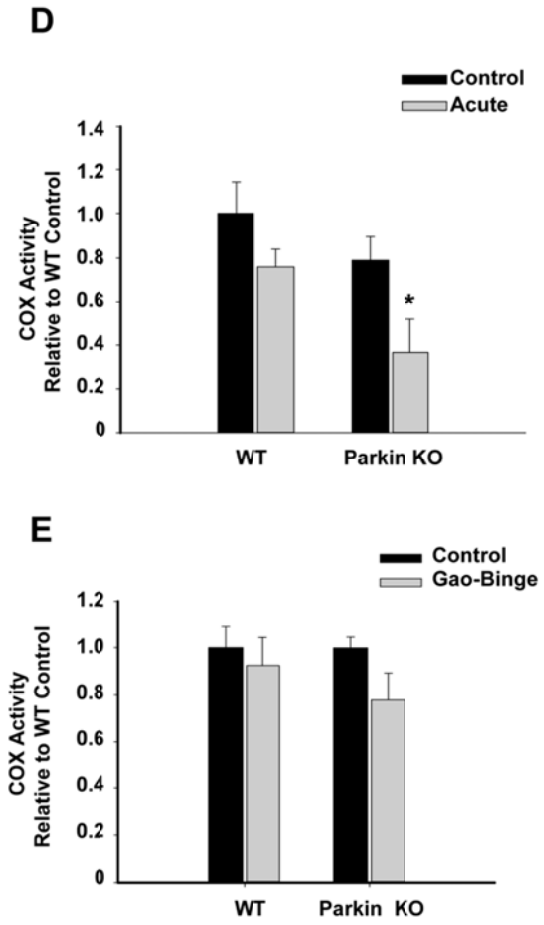

Figure 4.4.7. Mitochondrial respiration rates and COX activity were decreased in Parkin KO mice after alcohol treatment. A-C: WT and Parkin KO mice were treated with alcohol using the acute-binge model, and State 3 (**A**), State 4 (**B**), and maximal respiratory capacity (**C**) respiration rates were measured by Oroboros using isolated liver mitochondria. Data shown are means ± S.E. (n= ≥ 3 mice per group. *p<0.05 compared to WT alcohol by T-test). **D-E:** COX activity for WT and Parkin KO mouse livers treated with acute-binge (**D**) or Gao-binge (**E**). Data shown are means ± S.E. (n=≥ 3 mice per group. *p<0.05 compared to WT control by one-way ANOVA).

4.4.8 Parkin KO Mice had Increased Lipid Peroxidation after Alcohol Administration.

Alcohol is well known to cause oxidative stress and increased 4HNE staining as a result of increased lipid peroxidation in the liver. WT and Parkin KO mice had increased lipid peroxidation after both acute-binge and Gao-binge alcohol treatments, which was demonstrated by increased 4-hydroxynonenal (4HNE) staining (**Figure 4.4.8 A-D**). We also found that 4HNE staining occurred mainly in the peri-central vein areas in both WT and Parkin KO mouse livers. In addition, Parkin KO mice had increased lipid peroxidation compared to WT mice after alcohol treatment in both the acute-binge and Gao-binge models (**Figure 4.4.8 A-D**). Control levels of lipid peroxidation were similar between WT and Parkin KO mice (**Figure 4.4.8 A-D**). These results suggest that Parkin KO mice had higher levels of alcohol-induced oxidative stress compared to WT mice, which was likely due to the greater amount of alcohol-induced mitochondrial damage in Parkin KO mice compared to WT mice.

Figure 4.4.8

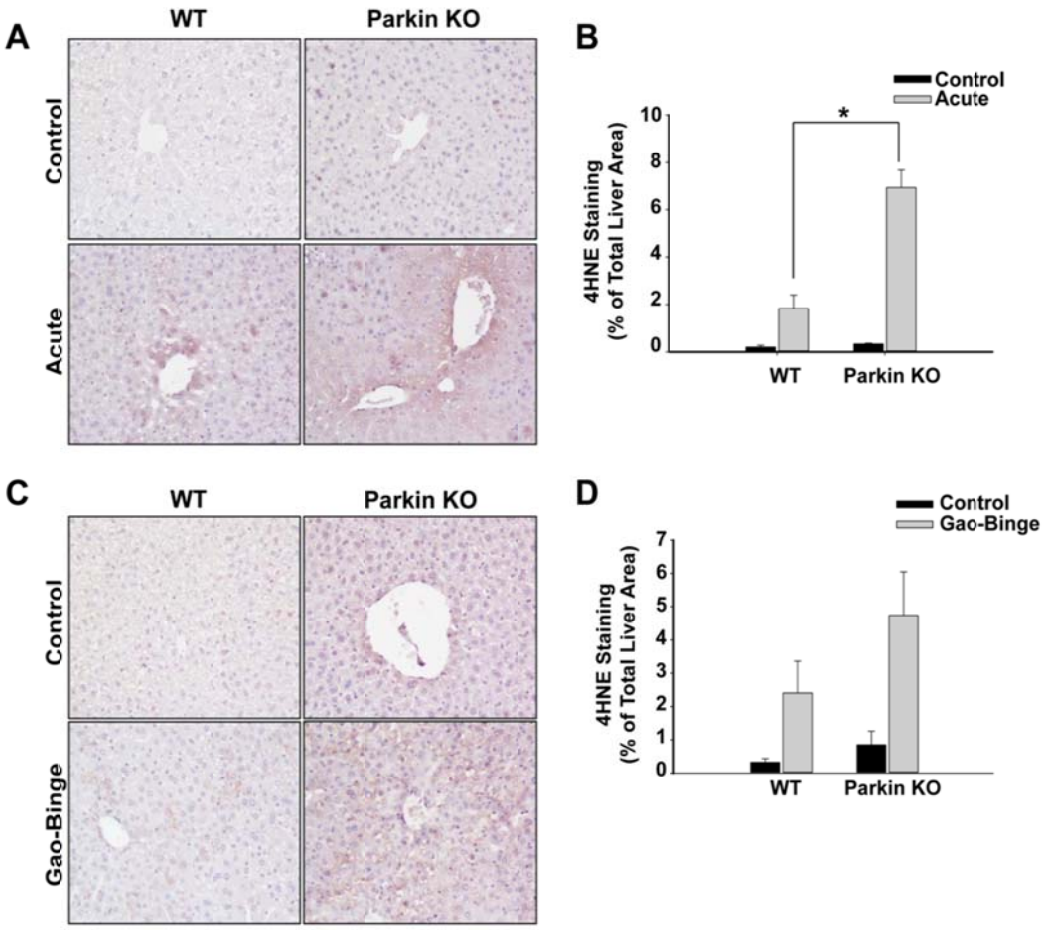

Figure 4.4.8. Lipid peroxidation was increased in Parkin KO mice compared to WT mice after alcohol treatment. A-D: WT and Parkin KO mice were treated with alcohol using the acute-binge (**A-B**) or Gao-binge (**C-D**) model. Representative images are shown from 4HNE immunohistochemistry after acute-binge (**A**) or Gao-binge (**C**) treatment, and data are represented as the percent (%) of positively stained areas compared to the total liver area (**B** (acute)**, D** (Gao-Binge)). Results shown are means ± S.E. (n=≥ 3 mice per group. *p<0.05 compared to WT control by one-way ANOVA).

4.5 Discussion

In this study, we found that Parkin KO mice had increased liver injury and oxidative stress after alcohol treatment compared to WT mice using both the acute-binge and Gao-binge alcohol models. In addition, we found that Parkin KO mice had increased steatosis in the acute-binge model compared to WT mice, but levels of steatosis were similar between WT and Parkin KO mice after Gao-binge treatment. Increases in liver injury and steatosis in Parkin KO mice were likely due to an increase in alcohol-mediated mitochondrial damage and dysfunction in Parkin KO mouse livers compared to WT mouse livers because Parkin KO mouse livers had severely swollen and damaged mitochondria that lacked cristae after alcohol treatment, which were not observed in WT mouse livers. In addition, Parkin KO mouse livers had decreased mitophagy, β-oxidation, mitochondrial respiration, and cytochrome c oxidase activity compared to WT mouse livers after acute-binge alcohol treatment. Decreases in Parkin KO mouse mitochondrial function were likely due to increased alcohol-induced mitochondrial damage and reduced mitophagy in Parkin KO mouse livers compared to WT mouse livers. In addition, mitochondria from Parkin KO mouse livers seemed less able to adapt to alcohol compared to WT mouse mitochondria resulting in severely damaged and swollen mitochondria in Parkin KO mouse livers. Furthermore, Parkin KO mouse livers had greater alcohol-induced oxidative stress-mediated lipid peroxidation compared to WT mouse livers. Therefore, our findings suggest that Parkin is an important protector against alcohol-induced liver injury and steatosis by maintaining mitochondrial integrity and function likely via mitophagy induction after alcohol treatment.

4.5.1 Parkin-mediated Mitophagy Likely Protects against Alcohol-induced Liver Injury

Mitophagy is a well-known protective mechanism for removing damaged mitochondria to prevent cell death and liver injury. For example, mitophagy has been shown to be protective

against apoptosis and injury in alcoholic liver disease (Ding, Li et al. 2010, Lin, Zhang et al. 2013) and also against necrosis in acetaminophen-induced liver injury (Ni, Bockus et al. 2012, Ni, Williams et al. 2013, Lin, Wu et al. 2014). However, the mechanism for how mitophagy is induced in the liver by alcohol is currently unknown. Parkin translocated to mitochondria after Gao-binge alcohol treatment in WT mice, suggesting that Parkin-induced mitophagy did occur after alcohol treatment. EM analysis showed that mitophagosome numbers were significantly increased in WT mouse livers, but not in Parkin KO mouse livers, after acute-binge and Gao-binge alcohol treatment compared to controls. These results suggest a possible defect in mitophagy induction after alcohol treatment in Parkin KO mice. Unfortunately, there is currently no reliable quantitative assay to quantify mitophagy *in vivo*, particularly in the liver. We could not detect any differences in LC3-II, p62, or mitochondria protein levels between WT and Parkin KO mice before or after alcohol treatment by Western blot analysis. This could be due to at least two reasons. First, alcohol-induced mitophagy could be mild in the mouse livers compared to other mitophagy models such as acetaminophen-induced mitophagy. Second, liver cells may adapt to alcohol-induced mitochondrial damage by activating mitochondrial biogenesis, which may offset the autophagic degradation of mitochondrial proteins as assessed by Western blot analysis.

It should be noted that alcohol could still induce mitophagy in Parkin KO mouse livers as assessed by EM studies, although the number of mitophagosomes was significantly decreased compared to WT mice. These results suggest that other compensatory mechanisms may exist for mitophagy induction in the liver in the absence of Parkin. There are several other proteins that have been shown to have important roles in the mitophagy pathway that may help compensate for loss of Parkin in the liver. For example, there are other E3 ligases such as Smurf1 and Mul1 that have been shown to have a role in mitophagy induction. In addition, Bnip3 Fundc1, and Nix have been shown to have roles in mitophagy induction during hypoxia, and the

inner mitochondrial phospholipid cardiolipin has also recently been shown to be able to induce mitophagy in neurons (Ni, Williams et al. 2015). Therefore, it is possible that one or several of these proteins are upregulated in the absence of Parkin to induce Parkin-independent mitophagy in the liver. However, the exact mediator of Parkin-independent mitophagy induction in the liver still needs further investigation.

Overall, Parkin-induced mitophagy was likely a protective mechanism in the liver after alcohol treatment because Parkin KO mice had reduced mitophagy levels and increased liver injury after alcohol administration compared to WT mice. However, we have only shown an associative relationship between reduced mitophagy and alcohol-induced liver injury in Parkin KO mice. Therefore, it is also possible that Parkin plays other roles in maintaining mitochondrial function in addition to mitophagy that may protect against alcohol-induced liver injury.

4.5.2 Acute-binge and Gao-binge Alcohol Treatments had Differential Effects on β-oxidation

We found that both the acute-binge and Gao-binge models caused hepatic steatosis, and increased steatosis was likely not due to increased fatty acid synthesis. Lee and colleagues also showed that acute-binge treatment for 24 hours did not result in increased fatty acid synthesis (Yin, Kim et al. 2007). The effect of the Gao-binge model on fatty acid synthesis has not been previously investigated. Interestingly, we observed that only the acute-binge model, but not the Gao-binge model, caused greater steatosis in the livers of Parkin KO mice compared to WT mice. These observations are associated with the different effects of the two alcohol models on β-oxidation. In the acute-binge model, several β-oxidation genes were induced in WT mouse livers after alcohol treatment but were left unchanged in Parkin KO mouse livers, suggesting that Parkin KO mouse mitochondria lacked the ability to induce β-oxidation as a protective mechanism. This inability to induce β-oxidation after acute-binge treatment likely led

to their increased levels of liver steatosis compared to WT mice. Gao-binge treatment did not cause induction of β-oxidation genes in either WT or Parkin KO mouse livers, suggesting that β-oxidation was inhibited in both WT and Parkin KO mouse livers after Gao-binge alcohol treatment. This may help to explain why the levels of steatosis were similar between WT and Parkin KO mice after Gao-binge alcohol treatment. Chronic alcohol feeding is well known to cause inhibition of β-oxidation, leading to accumulation of fat in the liver (Mantena, King et al. 2008). It seems that liver cells were able to adapt to alcohol-induced injury by increasing β-oxidation in acute-binge treated WT but not in Parkin KO mouse livers. However, this adaptation seemed to be lost after Gao-binge treatment. In addition to β-oxidation, the acute-binge and Gao-binge alcohol models may also dissimilarly affect fat uptake or secretion (Rasineni and Casey 2012), which could lead to differences in levels of liver steatosis after alcohol treatment between these models. Differences in alcohol-induced fat uptake and secretion between the acute-binge and Gao-binge models remain to be investigated.

4.5.3 Parkin KO Mice had Increased Numbers of Severely Damaged Liver Mitochondria and were Less Able to Adapt to Alcohol Treatment, Leading to Decreased Mitochondrial Function, Increased Oxidative Stress, and Increased Liver Injury and Steatosis

Parkin KO mouse livers had severely damaged and swollen mitochondria that lacked cristae after acute-binge and Gao-binge alcohol treatments, which were not seen in WT mouse livers. In addition, WT mouse livers had more elongated mitochondria after Gao-binge treatment, which is thought to be a cellular adaptive mechanism to chronic alcohol treatment (Han, Ybanez et al. 2012) , than Parkin KO mouse livers. Furthermore, Parkin KO mouse livers had greater levels of lipid peroxidation than WT mouse livers after alcohol treatment, which was likely caused by oxidative stress mediated by alcohol-induced mitochondrial damage. These

results all suggest that mitochondria in Parkin KO mouse livers were more damaged and dysfunctional compared to WT liver mitochondria after alcohol treatment.

Indeed, we found that WT mouse liver mitochondria had increased Complex I respiration rates while Parkin KO mouse liver mitochondria had decreased respiration rates after acute-binge alcohol treatment compared to controls. It has been shown that WT mice have increased respiration rates after oral alcohol feeding and intragastric infusion of alcohol, which was thought to be due to increased incorporation of respiratory complexes into the electron transport chain as an adaptation method (Han, Ybanez et al. 2012). However, Bailey and colleagues showed that State 3 respiration was unaffected by chronic alcohol feeding while State 4 respiration was increased (King, Swain et al. 2014). Differences in their results were likely due to the alcohol models or alcohol doses used. We observed increased respiration in WT mice treated with acute-binge for both State 3 and State 4 respiration, although it did not reach statistical significance. However, we found significantly decreased respiration in Parkin KO mice for State 3 respiration after acute-binge. Moreover, Parkin KO mice had decreased COX activity after acute-binge alcohol treatment compared to WT mice. These results suggest that Parkin KO mouse liver mitochondria were either more damaged by alcohol treatment or lacked the ability to adapt to alcohol treatment compared to WT mice. WT, but not Parkin KO, mouse mitochondria were likely attempting to adapt to alcohol treatment by increasing their respiration rates. It was previously shown that Parkin KO mice had decreased basal brain mitochondria respiration rates at 8 months of age compared to WT mice (Palacino, Sagi et al. 2004). We did not see any differences in basal liver respiration rates between WT and Parkin KO mouse livers at 2 to 3 months of age. It would be interesting to investigate if respiration rates are overall decreased in aged Parkin KO mouse livers and if this would have an impact on their alcohol-induced liver injury in the future.

Mitochondria are dynamic organelles that are well known to alter their fission and fusion rates in order to adapt to stress to maintain cellular survival. These fission and fusion events are necessary for cell survival because they allow the cell to adapt to changing conditions needed for cell growth, division, and distribution of mitochondria (van der Bliek, Shen et al. 2013). Mitochondria elongation was seen in chronic alcohol feeding and was suggested to be an adaptive response to alcohol treatment (Han, Ybanez et al. 2012). WT mouse livers had many elongated mitochondria after Gao-binge treatment, but Parkin KO mouse livers had less elongated and more swollen mitochondria than WT mouse livers. Parkin has been shown to play a role in regulating mitochondrial fusion and fission by promoting proteasomal degradation of mitofusin 1 and mitofusin 2 as well as Drp1 (Wang, Song et al. 2011, Ding, Guo et al. 2012). Future work is needed to determine whether these mitochondrial fusion and fission machinery proteins play a role in the elongated mitochondria observed in WT mouse livers exposed to alcohol. Nevertheless, these observations regarding mitochondrial morphology together with the mitochondrial respiration and COX activity data further support the notion that WT liver mitochondria seemed more able to adapt to alcohol treatment than Parkin KO mouse liver mitochondria. Overall, these data suggest that alcohol caused more damage to mitochondria in Parkin KO mouse livers than in WT mouse livers, which likely led to the increased liver injury and steatosis seen in Parkin KO mice compared to WT mice.

4.6 Conclusions

In conclusion, we found that alcohol caused more liver injury, oxidative stress, and steatosis in Parkin KO mice compared to WT mice, which was likely due to increased mitochondrial damage and dysfunction in Parkin KO mouse livers compared to WT mouse livers after alcohol treatment. This increase in mitochondrial damage and dysfunction in Parkin KO mice may have been partly due to decreased mitophagy in these mice. However, it is likely that Parkin may have other roles in maintaining mitochondrial function, such as regulation of

mitochondrial dynamics in addition to mitophagy, that are also important for protection against alcohol-induced steatosis and liver injury. The possible molecular events involved in Parkin-mediated mitophagy and mitochondrial functions for regulating alcohol-induced steatosis and liver injury are summarized in **Figure 4.4.9**.

Figure 4.4.9

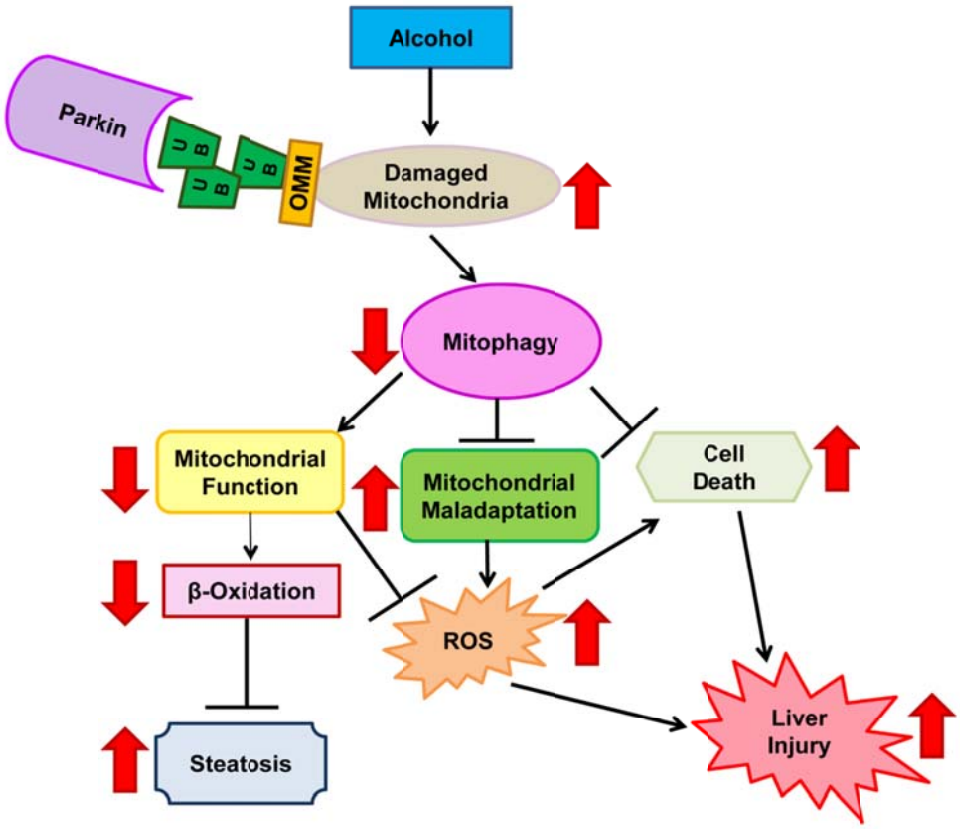

Figure 4.4.9. Summary of the role of Parkin in alcohol-induced steatosis and liver injury. Parkin is protective against alcohol-induced liver injury, oxidative stress, and steatosis by promoting mitophagy. Decreased mitophagy due to the absence of Parkin may lead to impaired mitochondrial function, decreased β-oxidation, increased ROS and lipid peroxidation as well as mitochondrial maladaptation (fewer elongated mitochondria and more swollen mitochondria) resulting in increased steatosis, cell death and liver injury after alcohol treatment. Consequences of Parkin loss after alcohol treatment are shown by red arrows.

Chapter 5. Overall Discussion and Future Directions

5.1 Discussion of Results

5.1.1 Parkin-induced Mitophagy Likely Protects against APAP and Alcohol-induced Liver Injuries, but Parkin is not Essential for Mitophagy Induction in the Liver.

We showed that Parkin-induced mitophagy is likely a protective mechanism in APAP and alcohol-induced liver injuries because Parkin translocated to mitochondria in WT mice after treatment with either APAP or alcohol. In addition, mitophagy was decreased in Parkin KO mice compared to WT mice after APAP or alcohol treatment. Furthermore, mice with acute knockdown of Parkin had greater liver injury after APAP treatment compared to WT mice, which was partly due to a significant decrease in mitophagy. Even though Parkin-induced mitophagy is likely a protective mechanism in the liver, we showed that Parkin is not essential for mitophagy induction in the liver because mitophagy occurred in Parkin KO mice after treatment with either APAP or alcohol. Mitophagy induction in Parkin KO mice was likely due to activation of adaptive mechanisms to compensate for the loss of Parkin. However, the Parkin-independent mechanisms of mitophagy induction in the liver are unknown and should be further explored.

There are several Parkin-independent mediators of mitophagy that may be activated during chronic loss of Parkin including NIX/BNIP3L, Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), Fun14 Domain containing 1 (FUNDC1), Cardiolipin, and Mitochondrial Ubiquitin Ligase 1 (Mul1). BNIP3 is a pro-apoptotic mitochondrial protein that contains a Bcl-2 homology 3 (BH3) domain (Yasuda, Theodorakis et al. 1998), and it induces mitophagy in hypoxic conditions and in starvation. BNIP3 is expressed in many tissues including the liver, but it is not highly expressed under normal conditions (Galvez, Brunskill et al. 2006). BNIP3 gene expression is induced during hypoxia by hypoxia-inducing factor-1 alpha (HIF-1α), which undergoes enhanced binding to its HIF-1-responsive element (HRE) on the BNIP3 gene promoter during oxygen deprivation (Bruick 2000). Mitophagy induction in MEF cells has been shown to be regulated by BNIP3 during hypoxia, which allows for removal of damaged

mitochondria and prevention of cell death via protection against ROS accumulation (Zhang, Bosch-Marce et al. 2008). Furthermore, phosphorylation of Ser17 and Ser24 in the LIR of BNIP3 was shown to initiate cell survival via induction of mitophagy by encouraging co-localization with LC3, suggesting that the phosphorylation status of BNIP3 decides whether it will behave as a pro-apoptotic protein or as a pro-survival protein by initiating mitophagy (Zhu, Massen et al. 2013).

NIX, also known as BNIP3L, is a homologue of BNIP3 (Chen, Cizeau et al. 1999). In addition to its roles in activating Parkin-mediated mitophagy (discussed in section 1.3.2), NIX can also activate mitophagy independent of Parkin. NIX activates autophagy by binding to Bcl-2, which dissociates the complex of Bcl-2 and Beclin-1, a protein necessary for initiation of autophagosome formation (discussed in section 1.2.1) (Bellot, Garcia-Medina et al. 2009). In addition, NIX interacts with the autophagosome membrane protein LC3 (Novak, Kirkin et al. 2010), and may therefore have a role in recruiting autophagosomes to damaged mitochondria. Like BNIP3, NIX plays a role in induction of mitophagy during hypoxia due to induction of its expression by HIF-1α (Bruick 2000). NIX and BNIP3 seem to have complementary roles in the mitophagy response during hypoxia because depletion of either NIX or BNIP3 did not affect autophagy, but depletion of both NIX and BNIP3 inhibited the autophagic response to hypoxic conditions. In addition, overexpression of both NIX and BNIP3 induced autophagy under normal oxygen concentrations (Bellot, Garcia-Medina et al. 2009). In addition to its role in mitophagy induction during hypoxia, NIX is also well known to induce mitophagy during red blood cell maturation. Red blood cells must eliminate their mitochondria during maturation in order to be able to better carry and provide oxygen, and NIX has been shown to be required for these cells to eliminate their mitochondria because mitochondrial entry into autophagosomes for degradation was blocked in NIX-deficient mice, which led to life-span reduction of red blood cells and development of anemia (Schweers, Zhang et al. 2007, Sandoval, Thiagarajan et al.

2008). This mechanism of mitophagy induction was also shown to require mitochondrial depolarization (Sandoval, Thiagarajan et al. 2008). NIX is also involved in mitophagy induction in cells that are undergoing high rates of oxidative phosphorylation, which leads to increased ROS production, mitochondrial dysfunction, cell death, and injury. When cells are undergoing high rates of oxidative phosphorylation, the GTPase Rheb is recruited to the outer mitochondrial membrane where it interacts with NIX and LC3 to induce mitophagy. This Rheb-dependent mitophagy pathway was shown to require NIX because depletion of NIX inhibited Rheb-induced mitochondrial degradation. Ridding of mitochondria undergoing high rates of oxidative phosphorylation via mitophagy helps maintain a healthy population of mitochondria, which promotes efficiency of cellular bioenergetics and mitochondrial energy production (Melser, Chatelain et al. 2013).

FUNDC1 is an outer mitochondrial membrane protein that also contributes to mitophagy induction under hypoxic conditions. Under oxygenated conditions, FUNDC1 is phosphorylated by the Src kinase on Tyr18 (Liu, Feng et al. 2012) and by casein kinase II (CK2). During hypoxia, the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) dephosphorylates FUNDC1 on Ser13 (Chen, Han et al. 2014). Hypoxia induces FUNDC1-dependent mitophagy using a different mechanism than NIX or BNIP3. While mRNA expression of NIX and BNIP3 are increased by HIF-1α during hypoxia, mRNA expression of FUNDC1 is actually decreased. When oxygen concentrations are low, phosphorylation of FUNDC1 is inactivated leading to an abundance of it in its dephosphorylated form. FUNDC1 contains an LIR, which allows it to interact with LC3 on autophagosome membranes. The binding affinity of FUNDC1 for LC3 increases when FUNDC1 is dephosphorylated (Liu, Feng et al. 2012). During hypoxia, CK2 is inhibited whereas PGAM5 interacts with FUNDC1 resulting in increased dephosphorylation of FUNDC1 to promote mitophagy (Chen, Han et al. 2014). Moreover, Bcl2-like 1 (BCL2L1), but not BCL2, interacts with and inhibits PGAM5, which prevents the

dephosphorylation of FUNDC1 and mitophagy (Wu, Xue et al. 2014). Furthermore, knockdown of FUNDC1 in HeLa cells prevented hypoxia-induced mitophagy (Liu, Feng et al. 2012), suggesting that FUNDC1 is required for mitophagy induction in hypoxic conditions. It is currently unknown which has a more important role in mitophagy induction during hypoxia, NIX or FUNDC1, but dephosphorylated FUNDC1 has greater binding affinity for LC3 than NIX (Novak, Kirkin et al. 2010).

Cardiolipin is a phospholipid dimer synthesized in the inner mitochondrial membrane that is able to translocate to the outer mitochondrial membrane upon mitochondrial damage and depolarization (Ren, Phoon et al. 2014). Cardiolipin initiates the mitophagy pathway upon externalization to the outer mitochondrial membrane in SH-SY5Y cells and primary cortical neurons after treatment with rotenone, which is an electron transport chain complex I inhibitor. Rotenone treatment increased GFP-labeled LC3 puncta, which is a marker of autophagosome formation. Rotenone treatment also increased LC3 co-localization with mitochondria, which is indicative of mitophagy induction. Prevention of cardiolipin translocation to the outer mitochondrial surface inhibited GFP-LC3 co-localization with mitochondria and mitophagy after rotenone treatment, (Chu, Ji et al. 2013), and inhibiting the co-localization of LC3 with mitochondria after rotenone treatment prevented autophagosome engulfment of mitochondria and degradation of the mitochondria in the lysosome (Chu, Bayir et al. 2014). However, cardiolipin and Parkin seem to mediate mitophagy under different levels of mitochondrial depolarization. For example, CCCP-treated cells caused Pink1 externalization to the outer mitochondrial membrane and recruitment of Parkin. However, Rotenone treatment only caused approximately 10% of mitochondria to depolarize, which caused cardiolipin translocation to the outer mitochondrial membrane but did not recruit Parkin. Cardiolipin translocated to the outer mitochondrial membrane after CCCP treatment, but not to the extent as during rotenone treatment, suggesting that there may be some overlap in Parkin and cardiolipin-induced

mitophagy pathways or that they both may respond to CCCP treatment (Chu, Ji et al. 2013). Interestingly, cardiolipin also functions to induce cellular apoptosis. If cardiolipin is present on the outer mitochondrial membrane in its peroxidized form, it initiates cell death through apoptosis. However, if it is not peroxidized, it initiates mitophagy to protect the cell from apoptotic cell death (Ren, Phoon et al. 2014). Chu *et al.* proposed that cardiolipin will initiate apoptotic cell death if the mitophagy pathway fails to degrade damaged mitochondria (Chu, Ji et al. 2013).

The E3 ligase Mul1 was recently discovered to act in parallel to Parkin for mitophagy induction by ubiquitinating the outer mitochondrial membrane mitofusin proteins in *Drosophila* and mammalian cell lines during mitochondrial depolarization. Overexpression of Mul1 in *Drosophila* reversed Parkin/Pink1 mutant phenotypes including mitochondrial clumping and elongated mitochondria. In addition, Pink1 and Mul1 or Parkin and Mul1 double mutant flies had worsened phenotypes than either mutant alone including increased mortality and muscle degeneration, reduced levels of ATP, and damaged mitochondria. In addition, Parkin KO and Mul1 knockdown primary cortical neurons had some increase in mitochondrial depolarization, but neurons from Parkin KO mice with Mul1 knocked-down had significantly increased mitochondrial depolarization and neuron degeneration. Mul1 was shown to act in a pathway independent of Parkin because knockdown or overexpression of Mul1 in Parkin-expressing HeLa cells did not affect Parkin translocation to mitochondria following mitochondrial depolarization (Yun, Puri et al. 2014). Therefore, Mul1 may be an important compensatory pathway during loss or inactivation of Parkin.

Any of these Parkin-independent mediators of mitophagy may be responsible for compensatory mitophagy induction in Parkin KO mice. For example, Bnip3 or Nix may mediate mitophagy in the absence of Parkin during APAP overdose because APAP overdose activates HIF-1α, which has been shown to induce Bnip3 and Nix expression, as previously discussed

(James, Donahower et al. 2006). Bnip3, Nix, or Fundc1 may also mediate mitophagy after alcohol treatment in the absence of Parkin because alcohol has been shown to cause hypoxia in the liver (Ji, Lemasters et al. 1982, Tsukamoto and Xi 1989, Arteel, Raleigh et al. 1996). Mul1 or cardiolipin may also have a role in mitophagy induction in Parkin KO mice after alcohol or APAP treatment because alcohol and APAP both induce mitochondrial depolarization (Kon, Kim et al. 2004, Masubuchi, Suda et al. 2005, Zhong, Ramshesh et al. 2014) (**Figure 3.4.7D**).

Interestingly, mitophagy was not greatly induced after alcohol treatment in Parkin KO mice by the acute-binge or Gao-binge models compared to controls (**Figure 4.4.5**), but it was significantly induced for both of these models in WT mice after alcohol treatment (**Figure 4.4.5**) and in both WT and Parkin KO mice after APAP treatment, although the rate of mitophagy was lower in Parkin KO mice (**Figure 3.4.3**). Our data suggest that Parkin plays a more dominant role than Parkin-independent molecules/pathways in triggering APAP-induced mitophagy. The differential roles of Parkin in alcohol and APAP-induced mitophagy could be due to the inability of compensatory pathways to induce mitophagy in the alcohol model. Mitophagy levels after alcohol treatment were much lower than after APAP treatment, so it is possible that compensatory pathways for mitophagy activation are not needed to compensate for chronic loss of Parkin after alcohol treatment. Alternatively, the compensatory mitophagy pathways in Parkin KO mice may not have been induced within our alcohol treatment period (acute and Gao-binge). It is unclear whether a longer chronic alcohol feeding would trigger compensatory mitophagy in Parkin KO mice. Therefore, it would be interesting to determine other mediators of mitophagy in the liver after APAP treatment in the future. Parkin KO mice with any of these other mitophagy mediators knocked down may provide evidence for one of these pathways acting in the absence of Parkin in the liver.

5.1.2 Parkin KO Mice had Opposite Responses to APAP and Alcohol-induced Liver Injuries

We demonstrated that Parkin KO mice had opposite responses to APAP and alcohol-mediated liver injuries because Parkin KO mice were protected against APAP-induced liver injury while alcohol treatment caused more injury in Parkin KO mice compared to WT mice. Parkin KO mice were protected against APAP-induced liver injury because they had decreased JNK activation along with increased Mcl-1 expression and hepatocyte proliferation compared to WT mice. Compensatory activation of Parkin-independent mitophagy pathways may have also had a role in protection against APAP in Parkin KO mice. Parkin KO mice had more liver injury and steatosis after alcohol treatment compared to WT mice because they had severely damaged mitochondria and decreased mitochondrial function after treatment with alcohol, which was likely due to decreased mitophagy. These opposing responses in Parkin KO mice to APAP and alcohol were likely due to Parkin having multiple roles in maintaining cellular homeostasis in addition to mitophagy (discussed in section 1.5).The importance of any of these Parkin-regulated pathways in response to liver injury may vary depending on the type and amount of damage an agent produces. For example, Parkin's roles in regulating cell cycle, JNK activation, and Mcl-1 expression were likely more important during APAP overdose than its role in mediating mitophagy, while Parkin's role in activating mitophagy was more important than other roles of Parkin during alcohol-induced liver injury.

The role that hepatocellular proliferation plays in repair could lead to differences in responses from Parkin KO mice after treatment with APAP or alcohol. There was massive hepatocellular necrosis in APAP-treated mice compared to mild cell death levels in alcohol-treated mice. APAP overdose is well-known to require a quick induction of hepatocellular proliferation and liver regeneration for survival (discussed in section 1.6.3). Even though liver regeneration is also required for repair from alcohol-induced cell death (Diehl 2005), current

alcohol models do not initiate enough cell death to require as large of a regenerative response as APAP overdose. Therefore, Parkin's role as a tumor suppressor (discussed in section 1.5.5) likely played a greater role in protection against APAP compared to alcohol, and increased proliferation in Parkin KO mice after APAP treatment may have overshadowed the detrimental effects of decreased mitophagy in these mice. To further confirm this, it would be interesting to determine if inhibition of proliferation in Parkin KO mice would increase their liver injury after APAP treatment.

In addition to proliferation, JNK activation and Mcl-1 expression have both been shown to have roles in APAP-induced liver injury. JNK activation exacerbates APAP-induced liver injury (discussed in section 1.6.2), while upregulation of Mcl-1 protects against APAP-induced liver injury (discussed in section 3.5.3). Parkin KO mice had decreased JNK activation and increased Mcl-1 expression compared to WT mice after APAP treatment, which allowed for their protection against APAP-induced liver injury. JNK activation by CYP2E1-induced oxidative stress has also been shown to have a role in alcohol-induced liver injury (Yang, Wu et al. 2012). However, differences in JNK activation were not evaluated between WT and Parkin KO mice after alcohol treatment. Based on APAP results, I would expect Parkin KO mice to have less JNK activation in the alcohol model compared to WT mice. However, this protective mechanism would likely not overcome the importance of Parkin's role in mitophagy induction after alcohol treatment because Parkin KO mice had increased injury after alcohol treatment compared to WT mice. In the future, JNK activation levels should be compared between WT and Parkin KO mice after alcohol treatment. If JNK activation is lower in Parkin KO mice than WT mice after alcohol treatment, then this would suggest that Parkin's role in mitophagy is a more important protective mechanism and possible therapeutic intervention strategy than manipulation of JNK in ALD. However, if JNK activation is higher in Parkin KO mice compared to WT mice after alcohol treatment, it would suggest different mechanisms of Parkin-mediated regulation of JNK

during APAP and alcohol-induced liver injuries and would reveal JNK activation as another mediator of alcohol-induced liver injury in Parkin KO mice in addition to decreased mitophagy and mitochondrial dysfunction. The role of Mcl-1 in ALD has not yet been determined. However, Mcl-1 is an anti-apoptotic protein that may also provide protection against alcohol-induced cell death. Therefore, future evaluation of Mcl-1 in ALD may also provide novel insight into mechanisms of alcohol-induced liver injury.

While other Parkin-mediated pathways showed greater importance after APAP treatment, mitophagy was likely the most important Parkin-mediated pathway necessary for protection against alcohol-induced liver injury and steatosis. Even though mitophagy was decreased in Parkin KO mice after both APAP and alcohol treatment, alcohol treatment caused less cell death compared to APAP and did not require an extensive regenerative response for repair, so decreased mitophagy was likely more detrimental in Parkin KO mice after alcohol treatment, leading to mitochondrial damage and dysfunction and increased liver injury and steatosis. Mitochondrial function was not evaluated in WT or Parkin KO mice after APAP treatment, but mitochondrial respiration has been shown to be decreased during APAP overdose (Meyers, Beierschmitt et al. 1988, Donnelly, Walker et al. 1994, Hanawa, Shinohara et al. 2008). Future investigation regarding differences in mitochondrial function after APAP treatment between WT mice and Parkin KO mice and between WT mice and mice with acute knockdown of Parkin would be interesting. Comparison of WT mice and mice with acute Parkin knockdown would be particularly interesting because mice with acute knockdown of Parkin had increased liver injury compared to WT mice after APAP treatment. I would expect mice with acute knockdown of Parkin to have decreased mitochondrial function because they had significantly decreased mitophagy after APAP treatment. If this is true, it may provide further evidence for the importance of mitophagy in protection against APAP-induced liver injury.

5.1.3 Whole-body Knockout and Acute Knockdown of Parkin had Differential Roles in APAP-induced Liver Injury

Whole-body Parkin KO mice and mice with acute knockdown of Parkin had opposite responses to APAP overdose. Parkin KO mice had decreased liver injury while mice with acute knockdown of Parkin had increased liver injury compared to WT mice after APAP treatment. In addition, Parkin KO mice had increased proliferation and Mcl-1 expression and decreased JNK activation while acute Parkin knockdown mice had decreased proliferation and Mcl-1 expression and increased JNK activation after APAP treatment. Opposing responses to APAP overdose in Parkin KO and acute knockdown mice were likely due to a lack of time for the acute knockdown mice to develop adaptive responses that were present in the Parkin KO mice. These results demonstrate that caution should be used when interpreting data from whole-body KO mice due to possible upregulation of compensatory pathways and adaptive responses. In addition, our results suggest that people with parkin mutations, such as patients with autosomal-recessive Parkinson's disease, may be resistant to APAP overdose compared to people with normal Parkin function. However, this has not been investigated.

5.1.4. Potential Therapeutic Options for APAP Overdose and ALD Through Modulation of Parkin Recruitment and Activation.

APAP-induced liver injury and ALD can both induce severe liver disease, for which there is no cure. Therefore, novel therapeutic interventions are needed to help prevent progression of these diseases. We have shown that Parkin-mediated mitophagy is still likely a protective mechanism in the liver, even though other compensatory mechanisms exist for mitophagy induction. Therefore, upregulation of the Parkin-mediated mitophagy pathway during alcohol or APAP-induced liver injury may be a beneficial therapeutic target to mediate removal of damaged mitochondria to prevent progression of liver injury. This was recently accomplished *in*

vitro by stabilizing Pink1 on the outer mitochondrial membrane via an ATP analog, kinetin triphosphate, which recruited Parkin to the mitochondria and prevented cell death in CCCP-treated Parkin-expressing HeLa cells (Hertz, Berthet et al. 2013). However, this has not been evaluated *in vivo*. In addition to targeting Pink1 stabilization, there are several other potential targets related to Parkin activation or its recruitment to mitochondria. For example, Parkin activation could also be increased by changing its auto-inhibiting conformation (discussed in section 1.3.2), but methods for changing the conformation of Parkin have not been developed. Inhibition of Pink1 kinase activity could also be a therapeutic target since Pink1 autophosphorylation and phosphorylation of Parkin and ubiquitin are needed for Parkin recruitment to mitochondria and for activation of its E3 ligase activity (discussed in section 1.3.2). However, this avenue for regulation of Parkin activity has not yet been explored. There are also several deubiquitinating enzymes that have been shown to regulate Parkin activity including USP15, USP30, and USP8. USP15 and USP30 are deubiquitinating enzymes that remove ubiquitin added to proteins by Parkin, thereby inhibiting Parkin-mediated mitophagy downstream of Parkin recruitment to mitochondria. Knockdown of both USP15 and USP30 were shown to enhance Parkin-mediated mitophagy and mitochondrial clearance in neurons and in *Drosophila* (Bingol, Tea et al. 2014, Cornelissen, Haddad et al. 2014, Cunningham, Baughman et al. 2015). USP8 removes ubiquitin chains on Parkin itself, which was shown to increase its mitochondrial translocation (Durcan, Tang et al. 2014). Therefore, modulation of these deubiquitinating enzymes may be another therapeutic target for activating Parkin-mediated mitophagy. However, targeting Parkin as a therapeutic option presents several problems due to the many roles Parkin plays in the cell in addition to mitophagy (discussed in section 1.5). In addition, therapeutics for inducing Parkin-regulated mitophagy would have to be directly targeted to the liver for treatment of ALD or APAP because Parkin is expressed in many tissues in addition to the liver including thymus, muscle, heart, brain, kidney, and spleen (Ding and Yin

2012). Another possible therapeutic target would be to up-regulate compensatory mitophagy mediators responsible for inducing mitophagy in the liver in the absence of Parkin (discussed in section 5.1.1). However, these pathways are not yet defined.

5.2 Future Directions

Parkin-independent mechanisms of mitophagy induction in the liver should be further investigated. A better understanding of mitophagy induction in the liver may allow for future development of novel therapeutic options for treatment of APAP overdose and ALD along with other forms of liver injury that involve mitochondrial damage and dysfunction. To determine Parkin-independent mechanisms for induction of mitophagy in the liver, acute knockdown of Parkin-independent mediators of mitophagy such as Mul1 or Bnip3 in Parkin KO mice should be performed, and these mice should be treated with APAP to determine if mitophagy is inhibited when potential compensatory pathways in addition to Parkin are blocked.

In addition, more critical evaluation of differences in mechanisms of injury between Parkin KO mice treated with APAP and alcohol should be conducted. JNK activation and Mcl-1 expression should be compared between WT and Parkin KO mice after alcohol treatment to determine if either of these pathways contributes to alcohol-induced liver injury. Mitochondrial function should also be compared between WT and Parkin KO mice in addition to comparison between WT mice and mice with acute knockdown of Parkin after APAP treatment to gain a better understanding regarding the role of mitochondrial function and mitophagy in protection against APAP-induced liver injury.

Finally, Parkin's role in liver regeneration should be better defined. Parkin KO mice had increased expression of the proliferative markers Cyclin D1 and PCNA before and after APAP treatment compared to WT mice. However, these markers are only suggestive of liver regeneration, so the role of Parkin in liver regeneration should be further evaluated using partial

hepatectomy. It would also be interesting to determine if inhibition of proliferation in Parkin KO mice would increase their liver injury after APAP overdose.

5.3 Concluding Remarks

We showed that Parkin translocated to mitochondria after APAP and alcohol treatments, and Parkin KO mice had reduced levels of mitophagy compared to WT mice. In addition, mice with acute knockdown of Parkin had greater liver injury after APAP treatment compared to WT mice, which was partly due to a significant decrease in mitophagy. Therefore, Parkin-mediated mitophagy plays a protective role against APAP and alcohol-induced liver injuries.

Interestingly we found that mitophagy still occurred, although to a lesser extent compared to WT mice, in Parkin KO mice after APAP treatments, suggesting that adaptive compensatory mechanisms for mitophagy induction exist in the liver during chronic loss of Parkin. We also revealed that Parkin KO mice had opposite responses to APAP and alcohol-mediated liver injuries, which was likely due to Parkin having multiple roles in maintaining cellular homeostasis in addition to mitophagy. In addition, our results suggest that data from evaluation of drug-induced liver injury mechanisms using whole-body knockout mice should be interpreted with caution due to the possible upregulation of adaptive and compensatory pathways in knockout mice. Overall, modulating Parkin-mediated mitophagy may be a promising therapeutic approach for targeting drug and alcohol-induced liver injuries.

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