The Regulation of Lipid Metabolism and Mitochondrial Quality Control in Health and Disease

by

Meghan Danielle Kapur

Department of Pharmacology and Cancer Biology
Duke University

Date: ______________________

Approved:

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Tso-Pang Yao, Supervisor

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Chris Newgard

___________________________

Raphael Valdivia

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Jeff Rathmell

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Matt Hirschey

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT
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Abstract

Demographics will dramatically shift in the next few decades as humans live longer. With this advancement in age comes an increase in the prevalence of diseases associated with aging. Incidence of obesity and type 2 diabetes correlates with increased age. Interestingly, canonical diseases of aging, such as Parkinson’s disease, also correlate with type 2 diabetes, suggesting an overlap of pathologies. Molecular commonalities include dysregulated lipid metabolism and mitochondrial quality control. Here, we analyze two players common to both pathologies.

Histone deacetylase 6 (HDAC6) is a cytoplasmic deacetylase that we have previously shown regulates critical stress-response pathways. Because loss of HDAC6 leads to defunct quality control, we hypothesized that this deficit would also affect metabolism. Indeed, loss of HDAC6 leads to aberrant fat accumulation in cells and in mouse liver. As HDAC6 is not a housekeeping gene, we tested whether HDAC6 KO mice would be more susceptible to diet-induced obesity, a clinically relevant stressor. After several weeks of high fat-diet, HDAC6 KO mice gain significantly more weight than WT counterparts on the same diet; they remain the same weight as WT mice on the control diet. As expected, we observe a concomitant decrease in glucose tolerance in HDAC6 KO mice on the high fat-diet regimen. To explain the excessive fat accumulation, we found that loss of HDAC6 disrupted intracellular metabolic processes: glucose uptake is increased while fatty acid oxidation is decreased in these cells. Altogether, our data point to an overall shift in metabolism that is reflected as increased lipid droplet accumulation.
In addition to lipid homeostasis, we wanted to investigate mitochondrial function in cells lacking HDAC6, as mitochondria are critical regulators of lipid metabolism. We have previously found that HDAC6 is critical for glucose withdrawal-induced mitochondrial elongation. Interestingly, glutamine withdrawal, which also promotes dramatic mitochondrial fusion, can still promote elongation in the absence of HDAC6. This data supports the notion that HDAC6 is a stress-response gene that responds to a very specific set of stressors.

Another stress-response gene critical for lipid homeostasis and mitochondrial function is the E3 ubiquitin ligase Parkin. Loss of Parkin in mice prevents diet-induced obesity and mutations in this gene lead to a familial form of Parkinson’s disease in humans. Lipids are also critical regulators of mitochondrial morphology and these dynamics influence Parkin’s ability to degrade damaged mitochondria. To this end, we hypothesized that perhaps lipid signaling was involved in the selective degradation of mitochondria, or mitophagy. Indeed, Parkin promotes accumulation of phosphatidic acid (PA) and diacylglycerol (DAG) in a chronological fashion. This effect is strictly Parkin-dependent as cells not expressing Parkin do not show accumulation of either lipid. Additionally, this process is actively controlled by Parkin. Expression of an ubiquitination-deficient mutant fails to promote DAG accumulation. We next asked whether lipin-1, a PA phosphatase that generates DAG, was critical for DAG accumulation and effective clearance of damaged mitochondria. Our data show that lipin-1 is critical for both; loss of lipin-1 prevents DAG accumulation and significantly blocks mitochondrial clearance. We also investigated another lipid-binding protein, endophilin B1 (EndoB1), with known roles in mitophagy. Loss of EndoB1 suppresses
DAG accumulation and mitochondrial clearance. Interestingly, loss of lipin-1 or EndoB1 halts mitochondrial clearance at the same point; proteasomal degradation of mitochondrial substrates is not affected, but autophagic degradation is disrupted. Altogether, this data points to lipid remodeling as a critical signaling pathway for clearance of damaged mitochondria.

HDAC6 and Parkin are necessary for effective clearance of damaged mitochondria. They are also important for lipid homeostasis under stress conditions. These conditions resemble stressors people experience in life, such as nutrient overload or chronic mitochondrial stress through aging. Here we aim to highlight the overlap between lipid metabolism and mitochondrial quality control and suggest how these pathways affect processes such as aging, obesity, and neurodegeneration.
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1. Introduction

1.1 Aging and disease

World life expectancy has more than doubled in the last two centuries (Oeppen and Vaupel, 2002). Prior to 1950, the increases in lifespan can be attributed to the reduction in early-life deaths while the latter half of the century saw increased care for those over 65 (Oeppen and Vaupel, 2002). Figure 1.1 shows the trends in life expectancy in the United States over the last few decades for the older population.

Figure 1.1: Life expectancy is increasing

Lifespan in Americans has increased steadily over the past century. Women consistently live longer than men. Note that life expectancy is increasing even in the elderly.

In the field of demographics, there is a contentious debate surrounding the lifespan of humans. One camp argues that we are rapidly approaching the maximum life expectancy, while the other suggests that we are not even close (Couzin-Frankel, 2011).

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Regardless of the maximum age, the evidence is irrefutable that our population is increasing and we are seeing an increase in lifespan (Colby and Ortman, 2015). Thus, the number of elderly people will expand dramatically in the next few decades (Figure 1.2). Previously, it was thought that increasing lifespan meant expansion of the time people are chronically ill or disabled. Due to advances in modern medicine, and societal factors such as education, the percent of disabled has decreased by approximately 2% in the last few decades (Mor, 2005). While this indeed is good news, the rate of increase in the elderly population will soon outweigh this decrease in disability. Therefore, it is critical that we address the chronic conditions facing our aging population. The most common illnesses that cause death in older citizens include heart disease, diabetes, and Alzheimer’s disease (NCHS, 2014).
**Figure 1.2: Aging population projection**

*Upper panel:* The age distribution of our current US population. *Lower panel:* Based on projection models, this graph illustrates the age distribution of our population in 2060. Note the large increase in the population ages 50-70. Additionally, the model predicts a significantly higher number of centenarians. Lavender: people born in the United States; dark purple: people born outside of the United States.

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Biological aging has been difficult to define. We observe the loss of weight, graying of hair, and cognitive decline in our aging population, but the biological basis for these and other changes is still controversial (reviewed in Newgard and Sharpless, 2013). What is not controversial, however, is that mitochondrial dysfunction has some part to play in this natural process. Indeed, mitochondrial dysfunction is one of the nine hallmarks of aging (reviewed in López-Otín et al., 2013) and will be discussed in greater detail in a later section.

1.1.1 Obesity and metabolic syndrome

Despite the linear increase in human life expectancy, demographers predict that we might observe a decline in lifespan due to the obesity epidemic (Mor, 2005). Approximately 35% of the United States’ population is currently overweight and another 28% are obese (Blackwell et al., 2014). Figure 1.3 illustrates the increases in obesity over the last several decades. This issue is not limited to just the U.S. In 2005, it was estimated that 1.1 billion people worldwide were overweight and another 312 million adults were obese (Haslam and James, 2005). Obesity is defined as accumulation of centrally localized fat, resembling an apple-shape as opposed to a pear-shaped body. To delineate between lean, overweight, and obese, clinicians use body mass index (BMI), or the ratio of a person’s mass and height: 1) lean/normal: 18.5-25, 2) overweight: 25-30, 3) obese: 30+ (Sturm et al., 2004). Exhaustive work has shown that adults who are overweight have a shortened lifespan of about three years; obese women live seven years less while obese men live six years less (Peeters et al., 2003). Not only does obesity shorten lifespan, but it also shortens active life expectancy, or lifespan without disability. This is quantified by surveying people for how many active daily-life chores they need help with, including bathing, eating, and dressing. For men, moderate obesity
(BMI 30-35) increases the chance of needing help by 50% and severe obesity (BMI 35+) increases this to 300%. The outlook is even worse for women: moderate obesity doubles this chance and severe obesity increases it to 400% (Sturm et al., 2004).

Figure 1.3: Percent of overweight and obese Americans over time

While the percent of adults that are overweight has remained relatively stable, there has been a dramatic increase in obesity and severe obesity for the last three decades.

This increase in disability amongst even our middle-aged population has an enormous effect on health-care spending. In 2008, the US spent about $113.9 billion on health care expenses associated with overweight and obese individuals. This amounts to 5-10% of annual health care spending (Tsai et al., 2011). Calculating this number is tricky due to a long list of comorbidities associated with obesity. Excess amounts of central fat lead to metabolic syndrome, or the concurrence of symptoms that result from increased weight circumference. The International Diabetes Federation developed an official definition of metabolic syndrome in 2005: increased waist circumference coupled

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with at least two of the following: raised triglycerides, decreased high-density lipoprotein (HDL) cholesterol, hypertension, raised fasting glucose, and/or type 2 diabetes (Alberti et al., 2005). Metabolic syndrome is therefore the convergence of high central adiposity, cardiovascular disease, and type 2 diabetes. Interestingly, a correlation between age and metabolic syndrome has been observed (Hildrum et al., 2007).

There are several hypotheses regarding why the obesity epidemic has exploded during the last few decades. Decreases in physical activity, increased diets of high-calorie/low-nutrient foods, and/or other environmental factors like processed foods are some ideas (Corkey and Shirihai, 2012). A genetic basis for obesity has also been examined. This field exploded in 1994 when the Friedman group cloned the leptin gene (Zhang et al., 1994), identifying it as mutated in genetically obese mice. Majority of the population does not have mutations in the leptin gene, but several genome-wide association studies (GWAS) have found numerous loci that track with increased central adiposity and BMI (Locke et al., 2015; Pei et al., 2014; Shungin et al., 2015). This would suggest that genetic susceptibility coupled with environmental factors have lead to the current obesity epidemic.

In addition to social, environmental, or genetic causes, research has intensively focused on the molecular basis of obesity and metabolic syndrome. Excess nutrients are stored as lipid in fat tissue. Because lipids can be modified and are hydrophobic, cells quarantine neutral lipid into storage organelles called lipid droplets. These structures are discussed in detail in a later section. Adipose tissue develops with a set number of adipocytes, or fat cells. When nutrient overload becomes too much, the fat must go elsewhere and the adipose tissue attempts to expand (reviewed in (Rosen and Spiegelman, 2014; Sun et al., 2011)). When it can no longer expand, the fat is
deposited in other tissues. This ectopic lipid storage coupled with inflammation is suggested to lead to systemic insulin resistance. In this way, excessive adipose causes cardiovascular disease due to extra lipid and cholesterol in the blood and type 2 diabetes because of insulin resistance (reviewed in (Taskinen and Borén, 2015)). More complex mechanisms exist beneath this simplified explanation as there are mouse models where the animal is obese but displays no signs of metabolic syndrome (reviewed in (Denis and Obin, 2013)). Therefore, studies outlining how lipid or nutrient excess leads to pathophysiological consequences are necessary to better understand the turning point from excess weight to metabolic syndrome.

1.1.2 Neurodegeneration: spotlight on Parkinson’s disease

1.1.2.1 Neurodegeneration

An expansion of our aging population is also increasing the number of people diagnosed with neurodegenerative diseases. These diseases are marked by substantial loss of specific populations of neurons leading to a variety of symptoms from cognitive to physical impairments (reviewed in (McEwen, 2007)). Aging is the number one risk factor for these diseases and due to their debilitating effects, we are facing even higher healthcare costs. Medicare costs were $555 billion in 2011 and this number is expected to reach approximately $903 billion in 2020 (CDC and US HHS, 2013).

Neurodegenerative diseases can be classified by the central nervous systems (CNS) they target or by the mechanism of disease pathology. Figure 1.4 shows the anatomical classification of some disorders. For mechanistic classification, there are five loose categories: trinucleotide repeat disorders, prion diseases, synucleinopathies, tauopathies, and generic protein aggregation (Przedborski et al., 2003). At the simplest level, these terms describe which proteins become aberrantly aggregated, believed to be
the cause of pathogenesis. Trinucleotide repeat disorders are caused by genetic expansions leading to excessive glutamine repeats within some proteins, and thus leading to aggregation. Prion diseases exhibit aggregation of a variety of proteins, but these are considered “contagious” inclusions because they can infect and damage nearby cells. Synucleinopathies and tauopathies are disorders caused by aberrant accumulation of synuclein and tau, respectively. Finally, other diseases exhibit generalized protein aggregation due to overwhelming stress from other systems. Figure 1.4 also illustrates the mechanistic classification of a range of neurodegenerative disorders.
Several neurodegenerative disorders are listed near the CNS region they affect. Alzheimer's disease is the aberrant accumulation of tau and β-amyloid proteins; it affects mainly the cerebral cortex (Lee and Leugers, 2012). General dementia has no definitive cause and also occurs in the cerebral cortex; there are, however, several types of dementia and some have mechanisms outlined. Fatal familial insomnia is a rapidly progressing genetic disease that initially affects the thalamus (Hughes, 1993). Progressive supranuclear palsy (inability of vertical eye movements), Huntington's disease (excessive, uncontrollable movement) and Parkinson's disease (inability to initiate voluntary movement) all begin in the basal ganglia (Przedborski et al., 2003). Amyotrophic lateral sclerosis (progressive loss of motor neurons) and spinal muscular atrophy (also progressive loss of motor neurons) affect the spinal cord (Przedborski et al., 2003). Lastly, Friedreich ataxia (impaired muscle coordination) begins in the cerebellum but migrates as it also affects the heart and spinal cord (Przedborski et al., 2003). Color of the disease name corresponds to mechanistic categories in box. See main text for description of mechanistic categories.

1.1.2.2 Parkinson's disease

Majority of neurodegenerative disorders are sporadic in nature; that is, there is no definitive genetic or environmental cause (Przedborski et al., 2003). Some, however, have a small percent that are genetic and Parkinson's disease is one of them, with less than 10% of cases originating from mutations (Bonifati, 2014). Parkinson's is the second-most common neurodegenerative disorder in the United States occurring in approximately 1-5% of the population (de Lau et al., 2004). It is the most common movement disorder and the risk of disease continues to rise with age. Canonical features of Parkinson's disease include: resting tremor, stooped posture, and shuffling gait. In addition to the physical symptoms, patients can also experience mood disorders, dementia and other cognitive impairments (Nussbaum and Ellis, 2003; Park and Stacy, 2009). The main cause of these symptoms is due to selective loss of dopaminergic neurons in the substantia nigra pars compacta (SN). The SN controls the initiation of voluntary movement, and dopamine neurons from this section project to several other regions in the brain, leading to the cognitive and mood problems. In 2002, the US spent
$23 billion in health care costs associated with Parkinson’s and models predict this figure could be as high as $50 billion in 2040 (Findley, 2007).

While symptoms from both sporadic and genetic forms of Parkinson’s disease resemble one another, they have separate pathologies. Sporadic Parkinson’s is the classical synucleinopathy, or the aberrant accumulation of α-synuclein inclusions called Lewy bodies. Post-mortem analysis demonstrates α-synuclein-positive aggregates in the SN of Parkinson’s patients (reviewed in (Goedert et al., 2013)). Not surprisingly, mutations in or multiplications of α-synuclein can lead to dominant forms of Parkinson’s. The function of α-synuclein is still not well understood, but it has been established that it is a lipid-binding protein and this could play a significant role in its pathogenicity (Outeiro and Lindquist, 2003; Willingham et al., 2003). There is more to the Parkinson’s puzzle than just α-synuclein, however.

In the late 1970s-early 1980s, separate incidences of illegal drug use became of importance for Parkinson’s disease researchers. In these cases, people obtained a street-batch of Demerol, a designer drug related to heroin, that was contaminated by N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (Davis et al., 1979; Langston, 1983). After ingesting the contaminated drug, patients became nearly immobile within a few days and displayed symptoms almost identical to Parkinson’s disease that became permanent. While these cases are tragic, they sparked a new avenue of research and MPTP administration is still used as a Parkinson’s model in rodents. MPTP enters the brain and is converted to MPP+ in neurons (Langston et al., 1984). MPP+ poisons the first complex of the mitochondrial electron transport chain (ETC), leading to altered respiration and increased reactive oxygen species (ROS) (Adams Jr et al., 1993; Nicklas et al., 1985). This evidence suggested that dopaminergic neurons in the SN were
particularly sensitive to mitochondrial disruptions. It also sparked research into whether pesticides and other environmental factors can lead to Parkinsonism, as MPTP is a naturally occurring compound. Indeed, several epidemiological studies have found a correlation between pesticide exposure and Parkinson’s disease (Betarbet et al., 2000; Moretto and Colosio, 2013).

In addition to the environmental evidence, autosomal-recessive forms of Parkinson’s exhibit a myriad of mutations in genes responsible for mitochondrial quality control. The two most commonly studied are PARK2 (Parkin) and PARK7 (PTEN-inducible kinase 1, PINK1) and were only discovered in the late 1990s (Abbas et al., 1999; Kitada et al., 1998; Lucking et al., 2000; Matsumine et al., 1997). Studies in flies demonstrated that loss of either PINK1 or Parkin lead to mitochondrial dysfunction in wing muscles; Parkin could rescue loss of PINK1, but PINK1 could not rescue loss of Parkin illustrating the epistatic relation of these two genes (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Since then, work analyzing PINK1 and Parkin function has exploded and their role in mitochondrial quality control will be discussed in a later section.

1.2 Simple and complex: lipid droplets and mitochondria

A common feature between obesity and Parkinson’s disease lies at the nexus of lipid metabolism and mitochondria. Here, we review basic information regarding the lipid-storage unit, lipid droplets, and the nuclear power-plant of the cell, mitochondria.

1.2.1 Lipid droplets

Traditionally, lipid droplets were thought to be inert storage vesicles for neutral lipid. Over the past few years, however, the importance and biology of these organelles has been realized (Beller et al., 2010; Farese and Walther, 2009; Fujimoto and Parton,
2011; Greenberg and Obin, 2008; Hashemi and Goodman, 2015; Walther and Farese, 2009). The droplet is a sphere of neutral lipid, namely triglycerides (TG) and cholesterol esters (CE), surrounded by a single phospholipid monolayer (Figure 1.5). Several proteomic studies have identified proteins that associate with lipid droplets ((Cermelli et al., 2006); reviewed in (Brasaemle, 2007; Wang and Sztalryd, 2011)). The main structural and functional proteins are the perilipin proteins, of which there are five. Perilipins 1 and 2 are always on lipid droplets and perilipins 3-5 can be either on lipid droplets or in the cytoplasm. The expression of these proteins is tissue-specific. Perilipins 1 and 4 are exclusive to adipose tissue, perilipins 2 and 3 are widely expressed, and perilipin 5 is primarily found in oxidative tissue (reviewed in (Sztalryd and Kimmel, 2014)).

![Diagram illustrates the basic structure of lipid droplets. A phospholipid monolayer surrounds a core of neutral lipid. Neutral lipids include triacylglycerols and cholesterol esters. Some cell types contain multiple lipid droplets (hepatocytes) while others contain a single, large lipid droplet (adipocytes).](image)

**Figure 1.5: Basic structure of lipid droplet**

Lipid droplets form as budding vesicles from the ER. Exactly how this is achieved and which elements regulate biogenesis is not completely known. However,
significant work has shown that lipid accumulates in between the phospholipid bilayer, followed by recruitment of lipid enzymes and local membrane remodeling (reviewed in (Pol et al., 2014)). In addition to tightly regulated biogenesis, lipid droplets can also fuse via the action of Fat-specific protein 27 (Fsp27, (Gong et al., 2011)), and this fusion is particularly critical for the formation of unilocular lipid droplets observed in adipocytes (Grahn et al., 2014). Lipid droplets are also actively transported throughout the cell mainly through a microtubule-dependent mechanism (Boström et al., 2005; Welte et al., 2005). It is hypothesized, however, that other cytoskeletal proteins are critical as the lipid droplet proteome revealed a number of cytoskeletal members (Zehmer et al., 2009).

Why do we care about lipid droplets? Mutations in genes necessary for lipid droplet formation lead to lipodystrophies, characterized by loss of adipose tissue (reviewed in (Garg, 2011)). An overabundance of lipid droplets leads to a different set of pathologies, namely obesity and metabolic syndrome. Ectopic accumulation of lipid in non-adipose tissues has been tightly correlated with insulin resistance (McGarry and Dobbins, 1999; Unger et al., 1999). However, simple lipid accumulation in skeletal muscle, for example, does not always correlate with pathologies. Obesity does lead to an abundance of lipid droplets in muscle tissue, but this is also observed in long-distance runners (reviewed in (Russell, 2004)). Clearly not all lipid droplets are equal and investigations into their dynamics will be critical for understanding metabolic syndrome and other lipid droplet-associated pathologies.

1.2.2 Mitochondria

Mitochondria are double membrane-bound organelles that coordinate cellular catabolism and anabolism. They have their own DNA (mtDNA), and transcription and translation machinery, which suggests that mitochondria were originally prokaryotes that were
engulfed by a eukaryotic cell (reviewed in (Zimorski et al., 2014)). In addition, they contain a host of metabolic pathways and the electron transport chain for energy generation. While typically thought of as static, bean-shaped structures, mitochondria undergo repeated cycles of fusion and fission. Pro-fusion proteins mitofusin 1 and 2 (Mfn1 and 2) are large GTPases that promote fusion between the outer-mitochondrial (OMM) membranes of two mitochondria (Chen et al., 2003), while optic atrophy 1 (OPA1) mediates fusion of the inner-mitochondrial membrane (IMM) (Olichon et al., 2003). To balance fusion, fission factors including fission 1 (Fis1; (James et al., 2003)) and dynamin-related protein 1 (Drp1; (Mozdy et al., 2000)) constrict mitochondrial membranes to split one mitochondrion into two. Figure 1.6 outlines these basic steps. Several types of stress affect mitochondrial morphology; nutrient withdrawal promotes elongation of mitochondrial tubules while uncoupling drugs lead to mitochondrial fragmentation (Gomes et al., 2011a). Disruption of these dynamics results in a host of pathologies (reviewed in (Youle et al., 2012)). For example, it has been shown in skeletal muscle that loss of mitochondrial fusion leads to decreased tolerance of mtDNA mutations (Chen et al., 2010). Mutations in OPA1 lead to dominant optic atrophy (Eiberg et al., 1994), and mutations in Mfn2 lead to Charcot-Marie-Tooth neuropathy type 2A (Züchner et al., 2004).
Figure 1.6: Basic structure of mitochondria and main players in fusion and fission
Diagram illustrating basic mitochondrial structure and molecular players involved in fusion and fission. A. Mitochondria have two phospholipid bilayers. Majority of metabolic pathways reside in the mitochondrial matrix. The ETC (electron transport chain) makes use of the IMM (inner mitochondrial membrane) to efficiently produce ATP. OMM: outer mitochondrial membrane; IMS: intermembrane space. B. Mitochondrial fusion requires two separate sets of machinery. Large GTPases Mfn1 and Mfn2 dimerize and bring together OMM. After OMM fusion, OPA1 dimerizes and promotes fusion of the IMM. C. Mitochondrial fission requires GTPase dynamin-related protein 1 (Drp1). Several Drp1 receptors exist including: fission 1 (Fis1), mitochondrial fission factor (MFF), mitochondrial dynamics protein of 49kDa (MID49) and of 51kDa (MID51). These proteins recruit Drp1 which then constricts and induces scission of the membranes.

Mitochondria are akin to nuclear power plants; they are highly efficient, but capable of producing toxic byproducts that can have catastrophic effects (Broadley and Hartl, 2008; Kageyama et al., 2012). It has been proposed that fusion and fission help mitochondria deal with these stresses. This can occur through separation of mitochondria, leading to quarantining damaged pieces of mitochondria from the healthier population followed by specific degradation of the deleterious piece (Twig et al., 2008). Therefore, understanding how cells regulate the quality of their mitochondria and deal with these catastrophic meltdowns will yield insight into diseases such as aging, obesity, and Parkinson’s.

### 1.3 Stress Response: Autophagy

Cellular stress-response pathways are critical to organismal survival. Here, we focus on a pathway necessary for a wide range of stresses, including starvation, proteotoxic stress, and mitochondrial damage ((Kim et al., 2008; Komatsu et al., 2006); reviewed in (Christian et al., 2013; Li et al., 2015; Yang and Klionsky, 2010)). Traditionally, autophagy, or “self-eating”, is viewed as a bulk process. An isolation membrane is initiated, engulfs cytosolic components, including organelles, and quarantines them in vesicles termed autophagosomes. Next, these vesicles fuse with
highly acidic lysosomes to break down its contents and release molecules necessary for critical biosynthetic pathways (see Figure 1.7; reviewed in (Tanida, 2011)). It has recently emerged that there are in fact several types of autophagy that can be highly specific. Two of these specialities are described below.

Figure 1.7: Steps of autophagy

Isolation membranes form around cytosolic components and begin to form the phagophore. Completely encircling the contents yields the autophagosome. Highly acidic lysosomes then fuse with the autophagosome forming the autophagolysosome, leading to degradation of its contents.

1.3.1 HDAC6 and quality control autophagy

Histone deacetylase 6 (HDAC6) is a cytoplasmic protein deacetylase with a variety of substrates including tubulin and F-actin-binding protein cortactin (Gao et al., 2007; Hubbert et al., 2002; Kovacs et al., 2005). Our group has shown that HDAC6-
mediated regulation of cortactin regulates the ability of autophagosomes to fuse with lysosomes under basal, but not starvation, conditions (Lee et al., 2010a). As such, our group proposed that HDAC6-mediated autophagy is for quality control purposes.

### 1.3.2 Parkin and mitophagy

Mutations in Parkin, an E3 ubiquitin ligase, were identified as a cause of autosomal recessive juvenile parkinsonism (AR-JP) (Abbas et al., 1999; Lucking et al., 2000). Work since then has outlined Parkin’s role in mitophagy, or the selective degradation of mitochondria. After damage, PTEN-inducible kinase 1 (PINK1) is expressed on the outer-mitochondrial membrane (OMM; (Jin et al., 2010; Meissner et al., 2011; Valente et al., 2004) where it phosphorylates, thereby recruiting and activating, Parkin and ubiquitin (Kane et al., 2014; Kazlauskaitė et al., 2014; Kondapalli et al., 2012; Koyano et al., 2014; Matsuda et al., 2010; Narendra et al., 2010a; Shiba-Fukushima et al., 2012; Vives-Bauza et al., 2010). After recruitment, Parkin ubiquitinates substrates on the OMM (Chan and Salazar, 2011; Glauser et al., 2011; Narendra et al., 2010b; Wang et al., 2011; Yoshii et al., 2011). This ubiquitination results in degradation of some OMM proteins and stimulates autophagy that selectively removes mitochondria (reviewed in (Yoshii and Mizushima, 2015)). Interestingly, we have also shown that HDAC6 is important in this process (Figure 1.8).
WT and HDAC6 KO MEFs were transfected with GFP-Parkin (green) and FLAG-HDAC6 (blue), treated with depolarization-inducing agent CCCP for 16h, followed by fixation and immunostaining with anti-Tom20 to visualize mitochondria. Note that WT MEFs clear mitochondria after sustained damage (top row), while loss of HDAC6 blocks this process (middle row). This effect is specifically due to HDAC6 as addition of exogenous HDAC6 rescues the phenotype (bottom row). Scale bars are 25um.

1.4 Thesis Goals

Increasing life span and the aging of the “Baby Boomer” generation has lead to an increase in aging-associated diseases. As our population continues to grow and modern medical advances promote increased life span, diseases of age will become a

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major health issue in the not-too-distant future. Therefore, we aim to investigate the pathology of two common age-associated diseases: obesity and Parkinson’s disease.

Using genetic models, we investigate the role of stress-response gene HDAC6. We aim to understand how HDAC6-mediated protein deacetylation influences metabolism and an organism’s ability to respond to metabolic stress. To this end, we analyze in vitro intermediary metabolism of HDAC6 KO cells to understand the mechanism of aberrant lipid accumulation. Additionally, we investigate the susceptibility of HDAC6 KO animals to diet-induced obesity.

Interestingly, HDAC6 is also critical in the mitochondrial stress-response. To this end, we investigate the role of lipid signaling during clearance of damaged mitochondria. Using microscopy and traditional molecular biology techniques, we analyze the role of Parkin, an E3 ubiquitin ligase, in promoting lipid remodeling during this stress response. We also suggest how this pathway could lead to preventative measures for diseases like Parkinson’s disease.

In studying HDAC6 and Parkin, we find an overlap between stress-response pathways dealing with nutrient overload and mitochondrial stress. Nutrient excess is the main cause of obesity while mitochondrial stress leads disorders such as Parkinson’s. Is there an overlap between metabolic disease and neurodegeneration? Our data suggests similar players and we aim to highlight the overlap.
2 The role of HDAC6 in fatty acid metabolism and mitochondrial quality control

2.1 Introduction

Protein acetylation has emerged as a regulator for major cell processes, specifically mitochondrial and intermediary metabolism (Choudhary et al., 2009; Wang et al., 2010; Zhao, 2010). We wanted to investigate whether protein deacetylase HDAC6 is critical in metabolic regulation. As the HDAC6 KO mouse is grossly normal (Zhang et al., 2008), we also wanted to ask whether HDAC6 is necessary under stress conditions, such as nutrient overload.

2.2 Results

2.2.1 HDAC6 opposes lipid droplet accumulation in vitro

As HDAC6 is an integral part of the cell’s quality control machinery, we decided to ask whether it also regulated other potentially stressful processes, such as intracellular metabolism. To this end, we investigated whether nutrient storage, namely lipid, was disrupted in mouse embryonic fibroblasts (MEFs) lacking HDAC6. As shown in Figure 2.1, we see an increase in lipid droplets in HDAC6 KO MEFs as marked by the neutral lipid dye Nile Red (green). As the HDAC6 KO mouse is grossly normal with no overt phenotypes (Gao et al., 2007; Zhang et al., 2008) we also wanted to investigate whether these cells could tolerate stress in the form of lipid overload, or a cellular version of high-fat diet. We treated these cells with oleic acid for 18hrs and again visualized lipid droplets via Nile Red staining (Figure 2.1, bottom panels). WT MEFs show a dramatic increase in lipid droplets after lipid loading, but the increase in HDAC6 KO MEFs is even greater.
2.2.2 HDAC6 opposes lipid accumulation in vivo

To investigate whether the in vitro lipid accumulation in HDAC6 KO MEFs has physiological relevance, we assessed the lipid content of livers from WT and HDAC6 KO mice. We chose this tissue because there is no obvious weight gain in the HDAC6 KO mice with standard chow over time (Zhang et al., 2008), and the liver is supremely
sensitive to whole body lipid status. As shown in Figure 2.2, we see a robust accumulation of lipid as evidenced by the neutral lipid dye Oil Red O (red) in HDAC6 KO mice.

![WT and HDAC6 KO mice](image)

**Figure 2.2: HDAC6 KO mice accumulate lipid in the liver**

Livers from WT and HDAC6 KO mice were isolated and frozen tissue blocks prepared in OCT compound. Sections were stained with Oil Red O, a dye that stains neutral lipid, and H&E. WT animals show a few lipid droplets basally, but there is an obvious and dramatic accumulation of neutral lipid in KO livers as evidenced by the increase in red signal indicating lipid droplets. Figure courtesy of Joo-Yong Lee.

Because we observe a dramatic increase in lipid droplets *in vitro* after lipid loading in HDAC6 KO MEFs, we hypothesized that putting these animals on a high-fat diet would yield a greater increase in lipid accumulation. To answer this question, we chose a very high-fat diet (VHFD, 60% kcal from lard) to ensure sufficient stress in order to parse out the role of HDAC6. Instead of using standard chow as the control, we chose a paired control diet that matched the sucrose levels found in the VHFD. This was to evaluate the effect of only increasing fat (and necessarily cholesterol), eliminating confounding phenotypes that result from sugar overload. Animals were started on the diet with their weights and the weight of the food consumed measured approximately weekly. As expected, HDAC6 KO mice gained significantly more weight than WT counterparts on the VHFD (Figure 2.3), while KO mice on the control diet did not differ from WT animals. The striking weight gain in HDAC6 KO mice was statistically
significantly different from control diet mice after just 4 weeks on the diet, while WT mice did not show a difference until 10 weeks (Figure 2.3, solid lines). Additionally, the KO animals were significantly heavier than WT mice on the VHFD at approximately 6 weeks, a whole month before the WT mice in this cohort gained more weight than control diet animals.

Figure 2.3: HDAC6 KO mice gain more weight faster than WT mice on VHFD

WT and HDAC6 KO mice were subjected to a control or a VHFD regimen (see Materials and Methods for diet details) for 16 weeks. The animals were weighed approximately every 7 days. Note that WT and HDAC6 KO mice on the control diet are the same weight. HDAC6 KO mice show significantly more weight gain than WT counterparts on the VHFD. Points represent means and error bars represent SEM. Statistical analysis was performed via two-way ANOVA using Tukey’s multiple comparisons test. #: significant difference between HDAC6 KO animals on the control versus VHFD; *: significant difference between WT and HDAC6 KO mice on the VHFD; #: significant difference between WT animals on the control versus VHFD.

With any weight gain study, it is critical to evaluate the feeding behavior of the animals. Does loss of HDAC6 affect food intake? To answer this question, we weighed
the food at each animal weighing and calculated the mass of food eaten per cage of mice. We then converted this mass to kcal as the caloric density of the control and VHFD are different (see Materials and Methods for details). As shown in Figure 2.4, there is no difference in calorie intake between treatment groups or genotypes over time. Therefore, the weight gain observed in HDAC6 KO mice on the VHFD does not result from an increase in energy intake. One caveat to this study is that we were not able to measure activity levels of the animals. Body weight is a reflection of the balance between calories eaten and calories burned. As such, we cannot rule out the possibility that the HDAC6 KO mice on the VHFD moved less than WT animals on VHFD, thus burning less fuel. We can surmise, however, that this is not a probable explanation for the excessive weight gain as the HDAC6 KO mice on the control diet displayed the exact same feeding behavior and weight gain as WT animals (Figures 2.3-2.4).
The amount of food eaten by each cage of mice was weighed every time the animals were weighed, yielding the mass of food eaten within the given time frame. A. Neither diet nor genotype affect energy intake over the course of diet regimens. *: significant difference between indicated sample and WT animals on the control diet after 1 week. B. The data from A were compiled to yield an average kcal taken in each day per mouse throughout the course of the treatment. Note there is no difference between any of the groups, illustrating that all genotypes and diet cohorts had the same energy intake. Points (A) or bars (B) represent means with SEM. Statistical analysis was performed using two-way ANOVA and Tukey’s multiple comparisons test.

Excessive weight gain has several detrimental effects on the body, the most well studied being disruption of glucose homeostasis. We hypothesized that since HDAC6 KO mice displayed even more weight gain than WT animals, their ability to clear a glucose bolus, or glucose tolerance, would be decreased. Decreased glucose tolerance
indicates a problem with insulin sensitivity and/or pancreas function. To assess the ability of these mice to clear glucose, we IP injected them with glucose and measured blood glucose levels over time. As shown in Figure 2.5, WT mice on VHFD cannot clear glucose as efficiently as either genotype on the control diet, as expected. Predictably, HDAC6 KO mice from the VHFD cohort had even lower glucose tolerance than WT animals. These animals had very high blood glucose levels (~315 mg/dL) as late as 120 minutes post injection. To better visualize the disruption of glucose clearance, the area under the curves in Figure 2.5A are plotted in Figure 2.5B. Clearly, the excessive weight gain in HDAC6 KO mice has detrimental effects to overall health of the animal.

**Figure 2.5:** HDAC6 KO mice on VHFD exhibit decreased glucose tolerance
Animals were injected with a bolus of glucose, followed by analysis of blood glucose levels at the indicated time points. A. Time course illustrating that glucose tolerance of WT and HDAC6 KO animals is the same on the control diet. Note that WT animals on the VHFD exhibit decreased glucose tolerance compared to the mice on control diets. HDAC6 KO mice show a dramatic glucose intolerance. B. The area of the curves shown in A. Points (A) or bars (B) represent means and error bars are SEM. Statistical analysis was conducted using two-way ANOVA with Tukey’s multiple comparisons test. #: significant difference between WT animals on control v VHFD; *: significant difference between HDAC6 KO mice on control v VHFD; ¥: significant difference between WT and HDAC6 KO animals on VHFD.

We previously observed lipid accumulation in the livers of HDAC6 KO mice with standard chow (Figure 2.2). A logical question to ask was whether VHFD affected the liver of HDAC6 KO mice differently from WT animals. Liver hypertrophy, or increase in size, is a hallmark of liver damage (reviewed in (Hall et al., 2012)). We therefore isolated livers from mice and weighed them immediately after collection. Because body weight also affects liver size, we normalized the mass of the liver to that of body weight to see if HDAC6 KO mice had liver hypertrophy. Interestingly, there was no difference in liver weight when normalized to body weight (Figure 2.6).

**Figure 2.6: HDAC6 KO mice do not show increased liver weight**

The liver of each animal was excised and weighed after dissection. The weight of each liver was normalized to the body weight of the mouse. While the actual mass of the livers from HDAC6 KO mice on VHFD was bigger than WT counterparts, this difference disappears when the body weight of the mouse is considered. Bars represent mean with SEM. Statistical analysis was performed using two-way ANOVA and Sidak’s multiple comparisons test.
An acetyltransferase partner to HDAC6, Mec17, was recently identified as regulating acetylation of tubulin (Akella et al., 2010; Kalebic et al., 2013; Kim et al., 2013). Additionally, one study found that acetyl-tubulin and Mec17 are necessary for adipogenesis in an in vitro model (Yang et al., 2013). We wanted to ask whether acetyl-tubulin or Mec17 was altered in the livers of our animals. Tissue was homogenized and analyzed via Western blot, as shown in Figure 2.7. Mec17 expression is fairly low under control diet conditions, but increases significantly after VHFD, even in HDAC6 KO mice. A concomitant increase in acetyl-tubulin is observed after VHFD in control animals. Because acetyl-tubulin levels are so high in HDAC6 KO mice (see the control diet lanes), we suspect a further increase in acetyl-tubulin is not detectable due to a saturation of tubulin acetylation basally. This data, in conjunction with our observation that HDAC6 KO mice have an accumulation in the liver basally suggests that acetyl-tubulin plays a role in liver lipid accumulation.

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**Figure 2.7: VHFD induces Mec17 and acetyl-tubulin levels**

Livers isolated from the animals described in Figures 2.3-2.6 were homogenized and whole cell lysates were run for Western blot analysis. Acetyl-tubulin levels are high in HDAC6 KO animals, regardless of diet, as expected. However, we observe a clear
increase in acetyl-tubulin levels after VHFD in WT animals. Interestingly, we also see an increase in Mec17 levels after VHFD, regardless of genotype.

2.2.3 Basic metabolic processes are disrupted in HDAC6 KO cells

The in vitro observation that HDAC6 KO MEFs show accumulation of lipid and this phenotype is recapitulated in animal models led us to further probe the mechanism behind this effect. We observed a greater accumulation of lipid droplets in HDAC6 KO MEFs after lipid loading (Figure 2.1), suggesting that lipid uptake is increased. Simple fatty acid uptake is not the only nutrient source that could result in an increase in lipid droplets. We therefore asked whether uptake of glucose, which can be converted to pyruvate, enter the TCA cycle, and be utilized for fatty acid synthesis, is also affected in these cells. To ask this question, we conducted a pulse-chase experiment utilizing radiolabeled 2-deoxyglucose, a non-metabolizable glucose analog. As shown in Figure 2.8, HDAC6 KO MEFs have a greater intracellular concentration of radiolabel compared to WT cells exposed for the same length of time. Cytochalasin B, a drug that depolymerizes actin and therefore blocks glucose uptake, was used as a negative control and affected both genotypes equally (data not shown).

![Figure 2.8: HDAC6 KO MEFs have increased glucose uptake](image)

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Radiolabeled 2-deoxyglucose was added to WT or HDAC6 KO MEFs and the amount of intracellular radioactivity was measured via scintillation counts. HDAC6 KO MEFs show an increase in glucose uptake compared to WT MEFs. Bars represent means with SEM. Student’s t-test (unpaired, equal variance) was used for statistical analysis.

As with body weight, the lipid droplet status is dependent upon opposing forces of nutrient uptake and intracellular catabolism. We have shown that nutrient uptake is enhanced in HDAC6 KO cells (Figure 2.1, 2.8). Is energy catabolism disrupted? To begin to answer this question we analyzed the ability of WT and HDAC6 KO MEFs to oxidize radiolabeled palmitic acid. Fatty acid oxidation generates water, which cells expel into the media. Using an ion-exchange resin column and eluting with water to obtain aqueous components, rather than the organic component that contains tritiated palmitate, we can separate between the radiolabeled molecules. Cells were incubated with radiolabel overnight, and conditioned media collected for analysis. Scintillation counts of tritiated water are shown in Figure 2.9. HDAC6 KO MEFs display a decreased ability to metabolize palmitate, suggesting that the accumulation of lipid droplets is due both to increased energy influx (Figures 2.1, 2.8) and to decreased catabolism (Figure 2.9). Etomoxir, an inhibitor of fatty acid transport into the mitochondria, was used as a negative control. Intermediary metabolism is a tightly controlled process with several layers of checks and balances. To assess whether the decrease in fatty acid oxidation was due to an inherent defect in the catabolic machinery, we also analyzed WT and HDAC6 KO MEFs that had been deprived of glucose overnight. As shown in Figure 2.9, removal of glucose restores fatty acid oxidation in HDAC6 KO MEFs. This illustrates that fatty acid oxidation as a process is intact, but the regulation of this pathway is deregulated upon loss of HDAC6.
Conditioned media was collected from WT and HDAC6 KO MEFs to measure scintillation counts for tritiated water that was produced through effective oxidation of the radiolabeled palmitate. HDAC6 KO MEFs show a decrease in fatty acid oxidation, but this effect can be reversed upon the removal of glucose. DS: dialyzed serum; Etx: etomoxir, inhibitor of fatty acid import into mitochondria; -Glucose: dialyzed serum in glucose-free DMEM (see Materials and Methods). Bars represent mean with SEM. Student’s t-test was used for statistical analysis.

2.2.4 HDAC6 affects mitochondrial morphology in response to glucose withdrawal

Due to the rescue-effect observed when HDAC6 KO MEFs were glucose-starved and fatty acid oxidation was restored, we also wanted to know whether other glucose-withdrawal phenotypes existed. In response to nutrient starvation, it has been reported that mitochondria will fuse in order to escape starvation-induced bulk autophagy (Gomes et al., 2011a; Rambold et al., 2011). We tested whether HDAC6 KO MEFs could adequately respond to glucose-withdrawal by elongating their mitochondria. We have

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previously reported that WT MEFs increase their mitochondrial connectivity upon glucose withdrawal while cells lacking HDAC6 do not (see Figure 2.11; different data set was published in (Lee et al., 2014)). We went on to show that HDAC6 deacetylates Mfn1, a pro-fusion GTPase, upon glucose-withdrawal and overexpression of an acetylation-mimicking Mfn1 mutant is sufficient to drive mitochondrial elongation (data not shown; see (Lee et al., 2014)). Importantly, the inability to sufficiently elongate mitochondria upon glucose withdrawal leads to an increase in reactive oxygen species (ROS) that cause mitochondrial damage (data not shown). In this way, HDAC6 acts as a stress responder by deacetylating, thus activating, Mfn1 after glucose withdrawal.

While we show in our previous work (Lee et al., 2014) that glucose starvation-induced deacetylation of Mfn1 stimulates mitochondrial fusion, we were curious to know how HDAC6 became activated under these conditions. AMPK, a well-characterized metabolic kinase, is activated upon an increase in the ratio of AMP/ATP (reviewed in (Hardie, 2014)), and thus decreasing anabolic processes in order to conserve ATP. Using MEFs lacking AMPK, we analyzed the mitochondrial morphology upon glucose withdrawal. Cells were immunostained and visualized via confocal microscopy. As seen in Figure 2.10, quantification of morphologies illustrates that dialyzed serum has no effect on WT or AMPK KO MEFs. Additionally, both WT and AMPK KO MEFs respond to glucose withdrawal by elongating their mitochondria. With both genotypes, the percent of cells exhibiting a hyperfused mitochondrial network increased after glucose removal. Therefore, energy-sensor AMPK is not responsible for upstream signaling promoting mitochondrial fusion after glucose removal. Identifying the HDAC6-activator will require further studies.
WT or AMPK KO MEFs were incubated in full, dialyzed serum (DS), or glucose-free (-G) media for 5 hours (see Materials and Methods). Both WT and AMPK KO cells show mitochondrial elongation after glucose starvation. This indicates that AMPK is not necessary for glucose withdrawal-induced mitochondrial fusion. Bars represent mean with SEM. Analysis was conducted using two-way ANOVA with Tukey’s multiple comparisons test.

It has recently been reported that addition of amino acids promotes shortening of mitochondria (Gomes et al., 2011b). We were curious if HDAC6 KO MEFs would fail to elongate mitochondria in response to other nutrient stresses. To this end, we chose to analyze mitochondrial morphology in the absence of glutamine. WT and HDAC6 KO MEFs were subjected to the nutrient withdrawal shown in Figure 2.11. Cells were immunostained to visualize acetyl-tubulin and mitochondria. In the upper panel, there is an obvious mitochondrial elongation in WT MEFs exposed to glucose-free, glutamine-free, or glucose- and glutamine-free conditions for 5 hours. In the lower panel, we see a failure of HDAC6 KO MEFs to increase mitochondrial fusion upon withdrawal of glucose. Surprisingly, however, we see a robust elongation after glutamine withdrawal (third row).

Figure 2.10: Loss of AMPK does not affect glucose withdrawal-induced mitochondrial elongation
The glutamine-starvation effect appears to be dominant as HDAC6 KO MEFs exposed to glucose- and glutamine-free media also exhibit an increase in connected mitochondria. This effect is quantified and plotted in Figure 2.12. There is a robust increase in the percent of HDAC6 KO MEFs with hyperfused mitochondria and a concomitant decrease in fragmented cells after glutamine removal. This evidence points to HDAC6 as a mediator in glucose withdrawal-induced, and not glutamine starvation-induced, mitochondrial elongation suggesting that the activation of fusion is not merely dependent upon generic starvation stress.
Figure 2.11: Glutamine withdrawal induces mitochondrial elongation in both WT and HDAC6 KO MEFs
WT or HDAC6 KO MEFs were incubated in full, glucose-free (-G), glutamine-free (-Gln), or glucose+glutamine-free (-G, -Gln) media for 5 hours. Cells were stained with DAPI (blue) to visualize the nuclei, anti-acetyl tubulin antibody (red), and anti-Tom20 (green) to see the mitochondria. **Top panel:** WT MEFs exhibit increased mitochondrial networking upon removal of glucose, glutamine, or both nutrients. **Bottom panel:** HDAC6 KO MEFs fail to fuse mitochondria after removal of glucose (second row). Surprisingly, these cells do elongate their mitochondria in response to glutamine removal (third row). The glutamine-withdrawal effect appears to be dominant as HDAC6 KO MEFs fuse their mitochondria upon removal of both glucose and glutamine (bottom row). Note the dramatic increase of acetyl-tubulin staining in the HDAC6 KO MEFs. Scale bars: 25um; zoom is 3x.

![Image of mitochondrial network](image)

**Figure 2.12:** HDAC6 KO MEFs can elongate mitochondria in response to glutamine withdrawal

Quantification of experiments conducted in Figure 2.11, above. WT and HDAC6 KO MEFs were subjected to glutamine starvation for 5 hours. The percent of cells with a hyperfused network increases significantly in both WT and HDAC6 KO MEFs. Note the significant decrease in percent of cells with fragmented mitochondria as well. Bars represent mean with SEM. Statistical analysis was performed using two-way ANOVA and Tukey’s multiple comparisons test.

### 2.3 Materials and Methods

#### 2.3.1 Immunofluorescence and confocal microscopy

All cells were maintained in DMEM (Gibco) with 10% FCS (Invitrogen) and 1% pen/strep (Gibco), with 5% CO₂ at 37°C. WT and HDAC6 KO MEFs were cultured on glass coverslips overnight, followed by treatment with DMSO or 250uM oleic acid (OA) for 18 hours. For nutrient withdrawal experiments, cells (WT, HDAC6 KO, and AMPK...
KO MEFS) were rinsed in the treatment media 3x before incubation. Samples were incubated in depleted media for 5 hours. Full media conditions were equivalent to maintenance media formulation. Dialyzed serum (Sigma, F0392) was supplemented in glucose-free DMEM (Invitrogen) or glutamine-free DMEM (Invitrogen) plus pen/strep for nutrient depletion studies. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 15 minutes, followed by rinsing in PBS. Next, cells were incubated in 0.15% Triton-X 100 in PBS, rinsed in PBS, and blocked for 20 minutes, room temperature in 10% BSA-PBS. Samples were incubated in primary antibody solutions overnight in a humidified chamber at 4°C. The following antibodies were used: anti-Tom20 (Santa Cruz, 1:500), anti-tubulin (Sigma, 1:500), anti-acetyl-tubulin (Sigma). The next day, coverslips were rinsed 3x for 5 minutes in PBS, incubated in secondary antibodies (Invitrogen or Jackson Immunoresearch) in 10% BSA-PBS for 20 minutes at room temperature, and rinsed again 2x for 5 minutes in PBS. For Nile Red staining, cells were incubated in 0.4 μM Nile Red (Invitrogen) in PBS for 1 hour, room temperature. Coverslips were briefly rinsed in PBS and mounted using Fluoromount G (Southern Biotech), with or without DAPI.

Slides were analyzed using a Leica SP5 confocal microscope. Z-stack images were obtained using the 100x/1.4-0.70 oil objective (11506210: HCX PL APO). Maximum projections are shown in the figures. Minor adjustments to brightness/contrast were done using ImageJ/Fiji, with no alterations to the Nile Red signal. White lines were drawn using ImageJ/Fiji’s ROI manager feature. Flattened images were used in the figure shown. Cropping images was done using Adobe Photosop.
2.3.2 Tissue Sectioning and Staining

Liver samples were embedded as frozen tissue blocks using OCT Compound (Tissue-Tek). Samples were cryosectioned and stained for H&E and Oil Red O following standard protocols. Images were acquired using a Zeiss Axio Imager microscope under brightfield light.

2.3.3 Animal Work

2.3.3.1 Animal husbandry, diet regimen, and weights

WT and HDAC6 KO mice (C57BL/6 and Swiss mixed background; (Gao et al., 2007)) began the diet regimens at ~6 weeks of age. Food was obtained from Research Diets. The VHFD (D12492, 5.24 kcal/g) contains the following: protein (20 kcal%), carbohydrate (20 kcal%), and fat (60 kcal%). In order to normalize the sugar content, a control diet (rather than standard chow) was used (D12450J, 3.85 kcal/g). The control diet contains the following: protein (20 kcal%), carbohydrate (70 kcal%), and fat (10 kcal%). While the carbohydrate values differ between the diets, the amount of sucrose in each is 17 kcal%. Animals were maintained on these diets for 16 weeks and weighed approximately every 7 days. At each weighing, the weight of the food was recorded and the weight of new food added was also recorded. This information, along with the number of animals in each cage, was used to calculate the food intake over time. As each diet had a different caloric density, the kcal/g ratio, provided above, was used to calculate the caloric intake for each cohort.

For analysis of liver weight, livers were excised from animals, rinsed in PBS, and immediately weighed. This value was normalized to the body weight of the mice measured immediately prior to sacrifice. All animal procedures were done in accordance with Duke University’s animal welfare policies.
2.3.3.2 Glucose Tolerance Test

Mice were IP injected with glucose (1 g/kg) and blood glucose measurements taken from a tail nick. Glucometer (Aviva) was calibrated prior to the experiment. Animals were monitored throughout the experiment for signs of hyperglycemia. Measurements were taken at time points indicated in Figure 2.5. Area under the curve (AUC) graph shown in Figure 2.5B was generated by calculating the trapezoid area of the curves depicted in 2.5A.

2.3.3.3 Liver whole cell lysate and Western blotting

After livers were weighed (outlined in section 2.3.3.1), they were placed in cryogenic vials and flash frozen in liquid nitrogen. For tissue homogenization, 100 mg of tissue sample was added to an eppendorf tube containing NETN lysis buffer and zinc oxide beads for use in the Bullet Blender Tissue Homogenization system (NextAdvance). After homogenization, NP-40 detergent was added to the samples, followed by normalization to protein concentration (BCA assay, Thermo-Fisher), addition of sample buffer (XT 4x Sample buffer, Biorad) and reducing agent, and boiling at 100°C for 5 minutes. Whole cell lysates were run on a 4-20% Tris-Glycine gel (BioRad), transferred to PVDF, and blotted using the following antibodies: anti-Mec17 (Sigma, 1:500), anti-acetyl-tubulin (Sigma, 1:5000), GAPDH (Cell Signaling, 1:2000). Membranes were developed using anti-mouse/rabbit-HRP (Promega) and ECL (PicoWest, Thermo-Fisher).

2.3.4 Radiolabeling: glucose uptake and fatty acid oxidation

2.3.4.1 Glucose Uptake

WT and HDAC6 KO MEFs were cultured in a 12-well plate overnight. Samples were rinsed with PBS 3x, followed by addition of KRH buffer (136mM NaCl, 4.7mM KCl,
1.25 mM CaCl₂, 1.25 mM MgSO₄, 10 mM HEPES, pH 7.4) containing radiolabeled 2-deoxyglucose (Perkin Elmer). After 20 minutes, samples were rinsed with PBS-glucose solution (with or without Cytochalasin B, Sigma, 40 uM), followed by lysis via .05% SDS buffer for 20-30 minutes at room temperature. 175 uL of whole cell lysate was used to measure scintillation counts of tritiated 2-deoxyglucose that was taken up into the cell. 2.5 mL of scintillation cocktail was added to the sample in scintillation vials and read on a Beckman-Coulter counter for 1 minute. The BCA assay (Thermo-Fisher) was used to measure protein concentration and the 3H CPM were normalized to ug of protein.

### 2.3.4.2 Fatty Acid Oxidation

This protocol was adapted from (DeBerardinis et al., 2006). WT and HDAC6 KO MEFs were cultured in duplicate 6 cm dishes. After ~10 hours from plating, the media was changed on each dish followed by the addition of radiolabeled palmitate (Perkin Elmer, NET043001MC) that was conjugated to essentially fatty acid-free BSA (Sigma) in PBS (ratio: 9%: 33.3%: 57.7%, respectively). Half of the samples also had etomoxir (Sigma, 100 uM) added as a negative control. The next day, 400 uL of media was collected, run over a Dowex pre-activated resin (1x80-200 ion-exchange resin, Sigma), and tritiated water eluted using deionized water. The scintillation counts were measured in a Beckman-Coulter counter. This procedure is outlined in greater detail in (Lee et al., 2014).

### 2.4 Discussion

We have found that cytoplasmic deacetylase HDAC6 plays a critical role in regulating nutrient balance, both in vitro and in vivo. We show that loss of HDAC6 leads to aberrant accumulation of lipid droplets in cells, and this effect is exacerbated upon lipid loading. Additionally, HDAC6 KO mice have an increase of lipid content in the liver. We stressed WT and HDAC6 KO mice with a very high-fat diet (VHFD) and show that
HDAC6 KO mice have greater weight gain than WT counterparts, but only on the VHFD. The livers of both WT and HDAC6 KO mice display an increase in tubulin acetyltransferase Mec17 levels after VHFD, suggesting that regulation of tubulin acetylation, or another overlapping, unknown substrate, is critical for management of lipid droplet levels. Finally, we probed at the basic mechanism for the in vitro lipid accumulation. We found that HDAC6 KO MEFs have an increase in glucose uptake and a decrease in fatty acid oxidation. Interestingly, the fatty acid oxidation machinery is intact as this phenotype is reversed upon glucose withdrawal.

An increase in basal glucose uptake, coupled with deregulated fatty acid oxidation suggests a compensatory metabolic mechanism in HDAC6 KO MEFs. Removal of glucose forces cells to utilize mitochondria as the main source of energy. As such, we asked what happened to the mitochondrial morphology of HDAC6 KO cells after glucose withdrawal. We found that loss of HDAC6 blunted glucose starvation-induced mitochondrial elongation and increased mitochondrial ROS. This information, coupled with our observation that fatty acid oxidation can increase in HDAC6 KO MEFs subjected to glucose removal, suggests that the metabolic state of mitochondria is not necessarily coordinated to their morphology. We previously reported that glucose withdrawal stimulates HDAC6-mediated deacetylation of Mfn1 to promote the fusion effect (Lee et al., 2014). To ascertain upstream signals that activate HDAC6 under these conditions, we analyzed mitochondrial morphology in WT and AMPK KO MEFs. We found that loss of AMPK did not prevent glucose withdrawal-induced mitochondrial elongation. Finally, we asked whether removal of glutamine, a critical amino acid for TCA cycle function and nitrogen replenishment, affected mitochondrial morphology. We found that both HDAC6 KO MEFs and WT counterparts exhibit robust fusion upon
removal of glutamine. This effect appears to be dominant over glucose-withdrawal, as a removal of both nutrients still promotes hyperfusion in HDAC6 KO cells. With this observation, we can separate between glucose starvation-induced and glutamine starvation-induced mitochondrial fusion.

Protein acetylation has emerged as a major regulatory post-translational modification, especially for mitochondrial and metabolic pathways (Choudhary et al., 2014; Wang et al., 2010; Zhao, 2010). Most studies outlining the role of protein acetylation in these pathways focus on the sirtuin (SirT) class of HDACs. SirT1 is critical in the fasting response, amongst other pathways, and SirT3-SirT6 are localized within the mitochondria where they can directly act on metabolic enzymes (reviewed in (Choudhary et al., 2014)). Our work outlining the role of HDAC6 in nutrient balance and mitochondrial health is novel in that other HDACs (classes I and II) have not been shown to have significant roles in intermediary metabolism. Additionally, we show that HDAC6 is critical for limiting intracellular lipid levels with nutrient excess and for stimulating stress-responses with nutrient withdrawal.

Excessive lipid intake is certainly a stress condition in people. It is known that susceptibility to obesity is inherited and several GWAS studies have found susceptibility loci to a variety of parameters, including BMI and fat distribution (Locke et al., 2015; Pei et al., 2014; Shungin et al., 2015; Speliotes et al, 2010). Here, we present HDAC6 as an obesity susceptibility gene candidate for predisposition to excessive weight gain. WT and HDAC6 KO animals on control diet are the same weight with similar glucose tolerances. However, after VHFD, the HDAC6 KO mice gain substantially more weight, with a concomitant decrease in glucose clearance, but with the same food intake as WT mice on VHFD. Our data demonstrate the vital role HDAC6 plays in lipid homeostasis.
3 Parkin promotes lipid remodeling at the mitochondrial surface during mitophagy

3.1 Introduction

Mitochondrial dysfunction has emerged as a causative factor of Parkinson’s disease (Matsumine et al., 1997). Parkin, which is mutated in some familial forms of the disease, promotes mitochondrial health by removing damaged organelles. Interestingly, loss of Parkin in mice prevents diet-induced obesity; the mechanism for this phenotype was suggested to be mediated by decreased fatty acid uptake and thus a blockade in fat accumulation (Kim et al., 2011). We therefore wanted to ask whether lipid homeostasis was also involved in Parkin-mediated mitophagy.

3.2 Results

3.2.1 PA and DAG accumulate on Parkin-positive mitochondria

It has been shown that mitochondrial dynamics are critical for effective mitophagy (Abeliovich et al., 2013; Twig et al., 2008) and that lipid remodeling on the mitochondrial surface can shape these dynamics (Huang et al., 2011). As such, we wondered whether this lipid remodeling pathway also occurred in Parkin-dependent mitophagy. To answer this question, we expressed a PA reporter plasmid (see Materials and Methods) and Parkin plasmid (either FLAG- or mCherry-tagged) in HeLa cells and treated them with 10uM carbonyl cyanide m-chlorophenylhydrazone (CCCP) to depolarize the mitochondria. As shown in Figure 3.1A, PA accumulates on Parkin-positive mitochondria as early as 1h after CCCP treatment. A greater percentage of cells exhibit PA accumulation after longer treatment times (Figure 3.3). This effect is strictly

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1 This chapter is being assembled into a manuscript: Meghan Kapur*, Kristi Norris*, Chun-Hsiang Lai, Latasha L Smith, Tso-Pang Yao. Parkin activates lipid remodeling to stimulate mitophagy.
dependent upon Parkin as cells lacking the E3 ligase do not exhibit PA accumulation, even after 18hrs of CCCP (Figure 3.1B). We next wanted to know whether DAG accumulated on damaged mitochondria after CCCP treatment. As shown in Figure 3.2A, DAG indeed accumulates on Parkin-positive mitochondria as early as 5.5hrs of CCCP treatment. Like PA, the percentage of DAG-positive mitochondria increases with time of treatment (Figure 3.3). Parkin is necessary for this effect as HeLas without Parkin do not show DAG accumulation on mitochondria, even after prolonged CCCP treatment (Figure 3.2B). Time course analysis (Figure 3.3) shows that the PA accumulation occurs before DAG.
Figure 3.1: PA accumulates on Parkin-positive mitochondria

HeLa cells were transfected with Parkin (blue) and PA-reporter (green, see Materials and Methods) plasmids, treated with DMSO or 10μM CCCP for the indicated times, and stained with anti-Tom20 to visualize mitochondria (red). A. PA accumulates on Parkin-positive mitochondria as early as 1 hour after CCCP treatment. B. PA does not accumulate in the absence of Parkin. Scale bars are 25μm; zoom is 3x.
Figure 3.2: DAG accumulates on Parkin-positive mitochondria

HeLa cells were transfected with plasmids for Parkin (blue) and DAG-reporter (green, see Materials and Methods). After DMSO or 10μM CCCP treatment, samples were fixed and stained for Tom20 to visualize the mitochondria (red). A. DAG accumulates on Parkin-positive mitochondria as early as 5.5 hours after CCCP treatment. B. This
accumulation does not occur in the absence of Parkin. Scale bars are 25um; zoom is 3x.

Figure 3.3: PA accumulation occurs before DAG

The percent of cells with Parkin-positive, lipid-positive mitochondria were quantified from experiments depicted in Figures 3.1-3.2. With DMSO treatment, neither PA nor DAG can be observed on mitochondria. Note the chronological accumulation of lipid; PA accumulates several hours before DAG. At least 50 cells per condition were counted in 2-3 separate experiments. Two-way ANOVA analysis was performed to determine statistical significance, with Sidak’s multiple comparisons test.

3.2.2 Pathogenic Parkin mutations affect PA and DAG accumulation

Our data illustrate that PA and DAG accumulation strictly depend upon Parkin. Because Parkin is mutated in AR-JP, we next wanted to ask whether pathogenic mutations affected PA and/or DAG accumulation. We first analyzed T240R and T415N Parkin mutants; these are defunct in ubiquitin E3 ligase activity (Hampe et al., 2006; Lee et al., 2010b). As shown in Figure 3.4A, PA accumulation does not require Parkin’s E3 ligase activity. Interestingly, DAG accumulation does (Figure 3.4B, C). From this evidence, lipid remodeling on the mitochondrial surface is indeed a regulated process dependent upon Parkin’s activity.
Figure 3.4: DAG accumulation requires Parkin’s E3 ubiquitin ligase activity

HeLa cells were transfected with mutant Parkin plasmids (blue) and lipid reporters (green). After 10uM CCCP treatment for 18 hours, slides were fixed and immunostained.
for Tom20 to visualize mitochondria (red). A. PA accumulates on Parkin-positive mitochondria regardless of Parkin’s E3 ligase activity. B. DAG accumulation is blunted in cells expressing E3 ligase-defunct Parkin mutants. C. The number of Parkin-positive cells with either PA or DAG accumulation. Despite efficient translocation, E3 ligase-deficient Parkin mutants fail to stimulate DAG accumulation. Scale bars are 25µm; zoom is 3x. At least 50 cells were counted in 2-3 independent experiments. Statistics were performed using two-way ANOVA and Sidak’s multiple comparisons test.

As the E3 ligase mutants do not have an effect upon PA accumulation (Figure 3.4), but Parkin is necessary for the effect (Figure 3.1), we next analyzed Parkin mutants that inefficiently translocate (Lee et al., 2010b; Matsuda et al., 2010). While translocation frequency is diminished, the R42P mutant is able to promote PA accumulation at 6hrs of CCCP treatment (Figure 3.5A). However, the K211N mutant displays ineffective PA accumulation (bottom row, Figure 3.5A) at early time points. At later time points, both mutants effectively promote PA accumulation (Figure 3.5B). Our evidence shows that PA accumulation requires Parkin translocation. When this translocation is inefficient, PA’s appearance on the mitochondrial aggregates is delayed, but not blocked. DAG accumulation is affected by K211N, but not R42P, however (Figure 3.6B). One final mutation we analyzed was R275W. This mutant has been shown to aggregate and become insoluble (Henn et al., 2005; Sriram et al., 2005). PA accumulation is reduced by approximately 50% (Figure 3.6A) with R275W. DAG accumulation is severely inhibited. Whether R275W delays full PA accumulation or indeed blocks it would require an extended time course study.
Figure 3.5: PA accumulation depends upon Parkin translocation

HeLa cells were transfected with Parkin mutant plasmids (blue) and PA-reporter plasmid (green). After CCCP treatment, cells were immunostained with anti-Tom20 to visualize mitochondria (red). A. Representative images are shown from 6h of 10uM CCCP treatment. Although R42P mutant Parkin (middle row) displays a lower frequency of translocation at 6h (data not shown), it can promote PA accumulation at levels equivalent to WT Parkin when it does translocate. K211N also exhibits decreased translocation frequency at 6h (data not shown) but fails to efficiently promote PA accumulation. B. Quantification of experiments shown in A. After 6h CCCP, K211N mutant Parkin cannot promote as much PA accumulation. However, by 18h K211N promotes the same amount of PA. Scale bars are 25um; zoom is 3x. Bars represent mean with SEM. Quantification is of at least 25 cells from 2 independent experiments. Statistical analysis conducted using two-way ANOVA and Tukey’s multiple comparisons test.
Figure 3.6: Additional Parkin mutants do affect PA and DAG accumulation

HeLa cells were treated as outlined in Figure 3.5. A. PA accumulation is decreased in cells expressing RING1 domain mutant, R275W Parkin. B. K211N and R275W mutants fail to promote DAG accumulation on Parkin-positive mitochondria. Quantification of at least 25 cells from 2-3 independent experiments is shown. Bars represent mean with SEM. Statistical analysis for A. student’s t-test and B. one-way ANOVA with WT Parkin as the control group.

3.2.3 Lipin-1 is necessary for DAG accumulation and efficient mitophagy

As PA can be converted to DAG at the mitochondria by the action of lipin-1 (Huang et al., 2011), we next wanted to ask whether lipin-1 is important for Parkin-mediated DAG accumulation. To answer this question, we generated a HeLa cell line stably expressing control or lipin-1-specific shRNA, transfected them with Parkin and PA- or DAG-reporters and treated the cells with DMSO or 10uM CCCP for 18h. As expected, PA accumulation is not affected by loss of lipin-1 (Figure 3.7). DAG accumulation is dependent upon lipin-1 as its loss blunts DAG accumulation (Figure 3.8). Thus, Parkin-mediated DAG accumulation is dependent upon lipin-1.
Figure 3.7: Lipin-1 KD does not affect PA accumulation

HeLa cells stably expressing control or lipin-1 shRNA were transfected with Parkin (blue) and PA-reporter (green) plasmids. Mitochondria were visualized using anti-Tom20 (red).

A. Both control and lipin-1 KD cells show effective PA accumulation on Parkin-positive mitochondria after 18h of 10uM CCCP. B. This observation is quantified from at least 25 cells in 2-3 independent experiments, showing no statistical difference. Scale bars are 25um; zoom is 3x. Bars represent mean with SEM.
Control or lipin-1 shRNA stably expressing HeLas were treated as in Figure 3.7. A. Control KD does not affect DAG accumulation on Parkin-positive mitochondria; loss of lipin-1 significantly reduces DAG accumulation. B. Quantification of images from A. Scale bars are 25um; zoom is 3x. Quantification is of at least 50 cells from 3 independent experiments. Statistical analysis was conducted using student’s t-test.

Our data shows that after Parkin translocates to depolarized mitochondria, phospholipid PA accumulates, followed by accumulation of DAG. The appearance of DAG on Parkin-positive mitochondria is regulated by lipin-1, and does so presumably...
through dephosphorylation of PA. The next logical question was whether lipin-1, and thus this lipid pathway, is necessary for mitochondrial clearance and mitophagy. To this end, we transfected our stable lipin-1 KD cells with GFP-Parkin, treated them with 10uM CCCP for 24h, fix and stained them for different mitochondrial compartments. To visualize the intermembrane space (IMS), we stained for cytochrome c, and to visualize the matrix, we stained for succinyl-CoA:3-oxoacid CoA transferase (OXCT1). HeLa cells are metabolically dependent upon glycolysis, and not mitochondrial energy production; thus, after 24h of sustained damage in Parkin-expressing cells, they will clear their mitochondria. Control KD cells exhibit effective clearance (Figure 3.9A, top row). However, upon loss of lipin-1, clearance is clearly blunted as mitochondrial aggregates are retained (Figure 3.9A, bottom row). To quantify this effect, we measured the cell area occupied by mitochondrial matrix (see Materials and Methods) and considered cells with less than 1% of their cell area occupied by matrix signal as cleared. Quantification confirms our observations (Figure 3.9B); mitochondrial clearance is indeed impaired after loss of lipin-1.

Efficient mitochondrial degradation via mitophagy occurs in two stages: proteasomal degradation of outer-mitochondrial membrane (OMM) proteins, such as the mitofusins (Mfns), followed by autophagic clearance of the remaining particles. In order to determine where lipin-1 regulates mitophagy, we analyzed which mitochondrial compartments were effectively degraded after loss of lipin-1. To achieve this, we transfected HeLa cells stably expressing YFP-Parkin with control or lipin-1 siRNA and treated them with 10uM CCCP for 24h. After 24h, control KD cells exhibit clear clearance of mitofusin proteins, known ubiquitination targets of Parkin, and Tim23, a marker for the inner-mitochondrial membrane (IMM, Figure 3.9C). Lipin-1 KD does not
block loss of Mfn1, but severely inhibits CCCP-induced degradation of Tim23. This effect was quantified (Figure 3.9D) from western blots of several different experiments. As proteasomal targets are degraded efficiently, our data show that loss of lipin-1 does not alter Parkin’s ability to direct proteasomal degradation. Blockade of Tim23 degradation indicates an interruption in autophagy-mediated clearance upon lipin-1 KD.

**Figure 3.9: Loss of lipin-1 decreases mitochondrial clearance and mitophagy**

A-B: HeLa cells stably expressing control or lipin-1 shRNA were transfected with GFP-Parkin (green) and treated with 10uM CCCP for 24h. After fixation, cells were stained with anti-cytochrome c (blue) for the IMS and anti-OXCT1 (red) for the matrix of the mitochondria. A. **Upper panel**: control KD cells exhibit effective mitochondrial clearance after prolonged CCCP treatment. Cells are outlined in white; note the significant reduction in mitochondrial signal from either compartment. **Lower panel**: lipin-1 KD cells show blunted mitochondrial clearance, as large mitochondrial aggregates are left after
prolonged depolarization. B. The cell area occupied by matrix signal (anti-OXCT1, red) from images acquired in A was measured (see Materials and Methods) and plotted. Cells with <1% of cell area occupied by matrix signal were considered to have cleared their mitochondria. Note the significant reduction in mitochondrial clearance after lipin-1 KD. C-D: HeLa cells stably expressing YFP-Parkin were transfected with control or lipin-1 siRNA, followed by overnight treatment with 10μM CCCP. C. Mitochondrial compartments were measured via Western blot. Mfn1 degradation is normal in lipin-1 KD cells, indicating that proteasomal degradation is not affected. However, Tim23 levels do not decrease after CCCP, indicating that lipin-1 is important for autophagy-mediated degradation of mitochondrial compartments after prolonged depolarization. Note the higher level of Mfn1 levels in lipin-1 KD cells. We suspect this is due to a decrease in basal mitophagy. D. Quantification of several western blots conducted as in C. Band densities were measured, normalized to loading controls, and then the change in protein level (levels with CCCP treatment – levels with DMSO treatment) plotted. Mfn1/2: either Mfn1 or Mfn2 was analyzed for each experiment. LpnKD: lipin-1 KD. For images, scale bar is 50um. For bar graphs, means are plotted with SEM. Student’s t-test (two-tailed, unpaired, equal variance) was used for each bar graph. C: Courtesy of Chun-Hsiang Lai.

3.2.4 EndoB1 is necessary for DAG accumulation and mitophagy

Endophilin B1 (EndoB1) is a lipid-binding protein with known roles in mitochondrial dynamics and autophagy (Karbowski et al., 2004; Takahashi et al., 2007). Additionally, it has been shown to promote the autophagic component of mitophagy (Takahashi et al., 2013), at the same point as lipin-1. Therefore, we wanted to ask whether EndoB1 was important for PA and/or DAG accumulation. We transfected HeLa cells with either control or EndoB1-specific shRNA, incubated them for 2-3 days, transfected with Parkin and PA- or DAG-reporter plasmids, and treated with 10μM CCCP for 18h. As shown in Figure 3.10, PA accumulation does not require EndoB1. However, DAG fails to appear on Parkin-positive mitochondria after EndoB1 KD (Figure 3.11).
Figure 3.10: Loss of EndoB1 does not alter PA accumulation
HeLa cells were transfected with control or EndoB1 shRNAs, incubated for 2-3 days, transfected with Parkin (blue) and PA-reporter (green) plasmids, and treated with 10uM CCCP for 18h.  A. Neither control KD nor EndoB1 KD cells exhibit disrupted PA accumulation.  B. Quantification of images shown in A. There is no statistical difference between conditions. At least 25 cells from 4 separate experiments were analyzed. Scale bars are 25um; zoom is 3x. Bars represent mean with SEM.

Figure 3.11: DAG accumulation requires EndoB1
HeLa cells were treated as in Figure 3.10. A. Control KD cells show efficient DAG accumulation on Parkin-positive mitochondria. Loss of EndoB1 dramatically reduces DAG accumulation after depolarization. B. Images from A were quantified. Note the marked reduction in DAG accumulation. At least 50 cells from 2-3 independent experiments were analyzed. Scale bars are 25um; zoom is 3x. Bars represent mean with SEM. Statistical analysis was done using student’s t-test (two-tailed, unpaired, equal variance).

Additionally, we show that EndoB1 fails to efficiently clear mitochondria (Figure 3.12A) and produces a blockade in degradation of autophagic, but not proteasomal, targets (Figure 3.12C). Intriguingly, this barricade exactly phenocopies lipin-1 KD.

![Figure 3.12: Loss of EndoB1 decreases mitochondrial clearance and mitophagy after prolonged depolarization](image)

A-B: HeLa cells were transfected with control or EndoB1 shRNA, incubated for 2-3 days, followed by transfection with GFP-Parkin (green). Cells were treated for 24h with 10uM CCCP, fixed, and immunostained for cytochrome c for the IMS (blue) and for OXCT1 for the matrix (red). A. Control KD cells exhibit clear loss of mitochondrial mass after prolonged CCCP treatment. Loss of EndoB1, however, dramatically affects mitochondrial clearance as large mitochondrial aggregates remain. B. Images from A were quantified by measuring the percent of cell area occupied by matrix (red) signal. Cells with <1% cell area occupied by mitochondrial matrix were considered to have cleared mitochondria. Note the significant reduction in mitochondrial clearance after EndoB1 KD. C-D: HeLa cells stably expressing YFP-Parkin were transfected with control or EndoB1 shRNA, incubated for 2-3 days, and treated with 10uM CCCP for 24h.
C. Western blot analysis illustrating similarities between lipin-1 KD and EndoB1 KD. Mitofusin degradation is normal in both groups, but there is a significant blockade in Tim23 degradation upon loss of EndoB1. This indicates that EndoB1 is critical for the autophagic component of mitophagy, in line with previous reports (Takahashi et al., 2013). Scale bars are 50um. Quantification of percent cell area occupied by matrix was done as in Figure 3.9. Statistical analysis was conducted using student’s t-test (two-tailed, unpaired, equal variance). C. Courtesy of Latasha L. Smith.

3.2.5 PA and DAG accumulate on mitochondrial aggregates in neuronal cells

The relevance of mitophagy in HeLa cells is debatable, especially in light of studies outlining that bioenergetic differences between HeLas and neurons prevents mass Parkin translocation after mitochondrial depolarization in the neurons (van Laar et al., 2011). Therefore, we wanted to investigate whether the lipid remodeling we observe on Parkin-positive mitochondria in HeLa cells also occurs in neuronal cells. We utilized human neuroblastoma cells, SH-SY5Y, that stably express FLAG-Parkin. After transfection of either PA- or DAG-reporters, we treated cells with 10uM CCCP for a variety of time points. As shown in Figure 3.13, both PA and DAG accumulate on mitochondrial aggregates. Furthermore, DAG accumulation appears after PA, suggesting that the lipid remodeling pathway we outlined in HeLa cells does occur.
Figure 3.13: PA and DAG accumulate on mitochondria in SH-SY5Y cells
SH-SY5Y cells stably expressing FLAG-Parkin (blue) were transfected (see Materials and Methods) with the indicated lipid reporters (green). Cells were treated with 10uM CCCP for the indicated times. After fixation, mitochondria were visualized by staining for matrix component OXCT1 (red). A. PA accumulates on damaged mitochondria as early as 3h after 10uM CCCP treatment. B. DAG accumulates on mitochondrial aggregates later than PA. C. Quantification of experiments from A and B. Note the time-dependent increase in PA and DAG accumulation. At least 25 cells from 2-3 independent experiments were counted for this quantification. Scale bars are 25um; zoom is 3x. Statistical analysis was performed using two-way ANOVA and Tukey’s multiple comparisons test.

3.3 Materials and Methods

3.3.1 Cell Culture and Microscopy

3.3.1.1 Cell culture, transfection, and immunofluorescence

All cells were maintained in DMEM (Gibco) with 10% FBS (Invitrogen) and 1% pen/strep (Gibco), with 5% CO₂ at 37°C. SH-SY5Y cells stably expressing FLAG-Parkin were a gift from the Lim group. Cells were cultured on glass coverslips, incubated overnight, and transfected with plasmids using XtremeGene 9 lipofection reagent. PA was visualized by Raf1-PABD-GFP (PA binding domain); DAG was visualized by CFP/YFP-DAGR (DAG reporter). Plasmids were a generous gift from the Frohman group. For siRNA, cells were treated with RNAiMax (Invitrogen) according to manufacturer’s instructions. For shRNA, cells were transfected using XtremeGene 9 according to manufacturer’s instructions. Cells stably expressing shRNA constructs (non-targeted and lipin-1 specific) were generated using standard retrovirus protocols and selected using puromycin. After transient transfection, media on cells was changed followed by treatment with DMSO or 10uM CCCP for indicated time points.

For immunofluorescence, cells were fixed in 4% paraformaldehyde for 15 minutes, followed by rinsing in PBS. Next, cells were incubated in 0.15% Triton-X 100 in PBS, rinsed in PBS, and blocked for 20 minutes, room temperature in 10% BSA-PBS.
Samples were incubated in primary antibody solutions overnight in a humidified chamber at 4°C. The following antibodies were used: anti-Tom20 (Santa Cruz, 1:500), anti-cytochrome c (BD Biosciences, 1:500), anti-OXCT1 (Proteintech Group, 1:250), anti-FLAG (M2, 1:500). The next day, coverslips were rinsed 3x for 5 minutes in PBS, incubated in secondary antibodies (Invitrogen or Jackson Immunoresearch) in 10% BSA-PBS for 20 minutes at room temperature, and rinsed again 3x for 5 minutes in PBS. Coverslips were mounted using Fluoromount G (Southern Biotech) without DAPI. Slides were analyzed using a Leica SP5 confocal microscope. Z-stack images were obtained using the 100x/1.4-0.70 oil objective (11506210: HCX PL APO).

3.3.1.2 Image Analysis

Maximum projections are shown in the figures. Minor adjustments to brightness/contrast were done using ImageJ/Fiji. White lines were drawn using ImageJ/Fiji’s ROI manager feature. Flattened images were used in the figure shown. Cropping images was done using Adobe Photoshop. To quantify the percent of cell area still occupied by mitochondrial matrix, we utilized a macro program written for ImageJ (Figure 3.14). Cells where mitochondria took up less than 1% of total cell area were considered to have cleared their mitochondria.
//duplicate image, crop, split channels, set measurements, choose green image
roiManager("Delete");
IJ.deleteRows(0, 50);
run("Duplicate...");
imageTitle = getTitle();
makeRectangle(0, 0, 510, 480);
run("Crop");
run("Split Channels");
run("Set Measurements...", "area perimeter integrated area_fraction limit redirect=None
decimal=2");
selectWindow(imageTitle + " (green)");
//adjust brightness of green image; hand-draw ROIs and add to ROI Manager
waitForUser("Adjust brightness/contrast of green channel. Draw ROIs and add to ROI
manager.")
selectWindow(imageTitle + " (blue)");
//run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.35");
setAutoThreshold("Default dark");
//run("Threshold...");
roiManager("Show all");
roiManager("Measure");
waitForUser("Ready for next channel?");
IJ.deleteRows(0, 50);
selectWindow(imageTitle + " (red)");
//run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.35");
setAutoThreshold("Default dark");
//run("Threshold...");
roiManager("Show all");
roiManager("Measure");
waitForUser("Done?");
run("Close All");
roiManager("Delete");
IJ.deleteRows(0, 50);

Figure 3.14: Macro program for ImageJ

We crafted a macro to automate mitochondria area measurements. Users hand-draw
around the cells of interest (in our case, Parkin-positive cells) and the program measures
OXCT1 signal within each region of interest (ROI).

3.3.2 Western Blotting

HeLa cells stably expressing YFP-Parkin (gift from the Youle group) were plated,
transfected with siRNA or shRNA as described above, expanded into 10cm dishes and
treated with DMSO or 10uM CCCP for 24hrs. After treatment, cell lysates were
collected by scraping cells into cold PBS, spinning, and resuspending pellet in 170mM
NaCl-NETN lysis buffer. Lysates were normalized to protein content using BCA assay.
Samples were reduced using XT buffer and reducing agent (Bio-Rad). Following lysis
and boiling, lysates were run on gradient gels 4-20% (Bio-Rad), transferred to nitrocellulose, blocked in 5% milk for 1 hr, and incubated with primary antibodies overnight in cold room. Primary antibodies used: anti-Tom20 (Santa Cruz, 1:1000), anti-Mfn1 (BD Biosciences, 1:1000), anti-Mfn2 (Santa Cruz, 1:1000), anti-actin (Sigma, 1:2000), anti-Tim23 (BD Biosciences, 1:1000), anti-EndoB1 (Imgenex, 1:1000), anti-lipin 1 (Cell Signaling, 1:750), anti-VDAC1 (Abcam, 1:1000), and anti-GAPDH (Cell Signaling, 1:2000). Membranes were developed using reagents and methods mentioned in Chapter 2.

3.4 Discussion

Here we show that Parkin promotes lipid remodeling on the mitochondrial surface. After depolarization, Parkin translocates to damaged mitochondria where it promotes PA followed by DAG accumulation (Figures 3.1-3.3). Pathogenic Parkin mutations affect this pathway (Figures 3.4-3.6). Importantly, loss of E3 ubiquitin ligase activity blocks DAG, but not PA, accumulation. Previous reports have outlined that PA can be converted to DAG by the action of lipin-1 at the mitochondria (Huang et al., 2011). Our results demonstrate that lipin-1 indeed is critical to this pathway; loss of lipin-1 has no effect on PA accumulation, but blocks DAG’s appearance on the mitochondria (Figure 3.7-3.8). Additionally, loss of lipin-1 interrupts mitophagy at the autophagic step, while not affecting proteasome-mediated degradation of mitochondrial components (Figure 3.9). Another lipid-binding protein, EndoB1, has roles in mitochondrial dynamics and mitophagy. We show that EndoB1 is necessary for DAG, but not PA, accumulation (Figures 3.10-3.11). Our data support previous reports that lack of EndoB1 blocks autophagic, but not proteasomal degradation, of mitochondrial targets (3.12). Finally, we
show that PA and DAG accumulate on mitochondrial aggregates in a neuronal cell line, illustrating that this pathway is relevant in neurons.

The initiation of Parkin-mediated mitophagy has been well characterized (Narendra et al., 2008, 2010a; Tanaka et al., 2010; Vives-Bauza et al., 2010). PINK1 activates Parkin, promoting ubiquitination of mitochondrial substrates and their proteasomal degradation. The specific mechanisms that occur after this point are murky. It has been proposed that ubiquitinated substrates bind scaffolding proteins such as p62 that recruit autophagosome components like LC3 (Geisler et al., 2010); this report, however, has been refuted (Narendra et al., 2010b). We show that there are regulated steps after proteasome-mediated degradation of OMM proteins that require Parkin-mediated ubiquitination. Additionally, this pathway is carried out through unique lipid remodeling at the mitochondrial surface.
4 Conclusions and Future Directions

4.1 HDAC6: lipid metabolism and mitochondrial dynamics

In Chapter 2, we discussed our observation that loss of HDAC6 leads to lipid droplet accumulation *in vitro* and in mouse liver; this effect can be exacerbated by lipid loading. In the case of mice, HDAC6 KO animals gain significantly more weight than WT counterparts on a very high-fat diet (VHFD) regimen. We also investigated glucose uptake and fatty acid oxidation in HDAC6 KO MEFs to determine which aspects of intermediary metabolism are disrupted. Glucose uptake is indeed higher upon loss of HDAC6 and fatty acid oxidation is reduced under basal conditions in these cells. Interestingly, the suppression of fatty acid oxidation can be completely rescued by the removal of glucose.

Our animal study illustrates the importance of HDAC6 in diet-induced weight gain. Several additional experiments would help to identify the mechanism by which HDAC6 acts. What is the cause of excess weight gain in HDAC6 KO mice? This is a complicated question as numerous pathways are critical for regulating body weight and energy homeostasis. However, by measuring energy intake vs energy output, we can begin to gain insight. We already show that HDAC6 KO mice do not consume more kcal per day compared to WT mice on either diet. Thus, HDAC6 does not affect appetite. A critical next step would be to measure activity levels of HDAC6 mice. We would hypothesize that HDAC6 does not affect activity level, however, because these mice are equal in weight to their WT counterparts while on the control diet. Another important measurement would be fat content of the fecal matter of these animals to evaluate whether loss of HDAC6 promotes excessive lipid absorption through the intestine.
Evaluating lipid and cholesterol blood profiles would also indicate whether there is an excessive amount of circulating lipid entering the blood stream.

Where do HDAC6 KO mice gain weight? We suspect that the excessive weight gain occurs in the adipose tissue of HDAC6 KO animals, but this should be specifically measured using a DEXA scan. Furthermore, it has been well established that fat depots do not equally promote metabolic syndrome, and, in some cases, transplanting subcutaneous adipose to visceral depots helps to resolve insulin resistance (reviewed in (Lee et al., 2013a)). Which fat pads, if any, are enlarged upon loss of HDAC6? Our work demonstrates that VHFD induced Mec17 levels and acetylated-tubulin in the livers of WT and HDAC6 KO mice. This suggests that HDAC6 is activated only under stress conditions, in this case nutrient overload, in an attempt to counterbalance activation of Mec17. Critical next steps would include analyzing activation of this pathway in other tissues. It has previously been reported that Mec17 and acetylation of tubulin are necessary for adipogenesis (Yang et al., 2013). It is also interesting to note that Mec17 has recently been shown to interact with clathrin-coated pits (Montagnac et al., 2013) and these structures, which are positive for caveolin-1, are critical for lipid droplet homeostasis in adipose tissue (Cermelli et al., 2006; Ostermeyer et al., 2001). We would hypothesize that increased acetylation of tubulin at clathrin-coated pits might promote lipid accumulation.

Finally, it has recently been shown that regulation of mitochondrial dynamics in specific neuronal populations regulates whole-body energy homeostasis. Interestingly, loss of mitofusin proteins in Agrp neurons blocks diet-induced obesity (Dietrich et al., 2013). In this neuronal population, excess nutrients actually promote mitochondrial elongation. Is this the same mechanism as glucose or glutamine withdrawal-induced
mitochondrial elongation? Loss of Mfn2 in POMC neurons, the balance to Agrp neurons, exacerbates obesity resulting from high-fat diet (Schneeberger et al., 2013). Clearly, mitochondrial dynamics play a critical role in metabolic homeostasis, but this heavily depends upon cell type. It would be interesting to investigate whether the role HDAC6 plays in mitochondrial dynamics also contributes to the excessive weight gain observed in KO animals. For example, thermogenesis in brown fat requires intact mitochondria and is another form of energy expenditure. Perhaps thermogenesis is disrupted due to stressed mitochondria, leading to the increased weight gain because fewer calories are burned.

4.2 Parkin, lipid, and mitochondrial quality control

In Chapter 3, we outlined a pathway whereby Parkin promotes phospholipid PA accumulation on damaged mitochondria. DAG accumulation is observed several hours after PA, supporting the role of PA phosphatase lipin-1 in this process. Additionally, pathogenic mutations in Parkin can block the appearance of PA or DAG at specific points. Namely, Parkin’s ability to translocate is required for PA accumulation and its E3 ubiquitin ligase activity is necessary for DAG accumulation. We also show that lipid-binding protein EndoB1 is critical for DAG accumulation. Our data together point to a regulated pathway downstream of Parkin’s ubiquitination activity that is independent from proteasome-mediated degradation. This is also of interest because there is a substantial amount of evidence linking lipid dynamics to other Parkinson’s disease pathological players, such as α-synuclein, which is discussed below.

The PA to DAG conversion via lipin-1 at mitochondria has previously been reported (Huang et al., 2011), but not in the context of Parkin-mediated mitophagy. In this report, the authors identified mitochondrial phospholipase D (mitoPLD), which
converts cardiolipin to PA, as the source of local PA production. Does mitoPLD also play a role in Parkin-mediated mitophagy? We would analyze PA and DAG accumulation upon loss of mitoPLD to answer this question. Another group has shown that flipping cardiolipin from the inner-leaflet of the OMM to the outer-leaflet by the action of phospholipid scramblase 3 (PLSCR3) can recruit LC3 to the mitochondria (Chu et al., 2013). LC3 is a canonical marker for autophagosomes. Is this pathway also critical in Parkin-mediated mitophagy? If cardiolipin could not be flipped to the outer-leaflet, would that block PA accumulation? Answering these questions would shed more light on how Parkin stimulates this lipid remodeling. Finally, how does DAG promote mitophagy? Previous studies suggest that DAG activates atypical protein kinase c (Zhang et al., 2014) in order to initiate autophagy. More work would need to be done in order to verify this is the mechanism for Parkin-mediated mitophagy. Completely understanding Parkin function certainly benefits patients with pathogenic mutations leading to AR-JP and helps us better understand mitochondrial quality control mechanisms.

Mitochondrial function and lipid droplet homeostasis are tightly linked. Mitochondria cannot synthesize all of its own lipids, so it imports them through physical connections to the ER. These connections are termed mitochondria-associated membranes (MAM; reviewed in (Vance, 2014)). Defects in MAM result in disrupted mitochondrial dynamics and calcium homeostasis (reviewed in (Marchi et al., 2014)). It is tantalizing to speculate that these connections would also be capable of initiating or producing lipid droplets in response to mitochondrial needs. Mitochondrial stress, for example, promotes lipid droplet formation (Lee et al., 2013b), and perilipin 5 has been shown to associate with lipid droplets and mitochondria (reviewed in (Wang and Sztalryd, 2011)). Whether the MAM is critical for lipid droplet formation basally or after
stress requires future study. Additionally, PA and DAG are important components of lipid droplet monolayers. They promote negative curvature of the membrane that facilitates stability when the lipid droplet is budding from the ER (reviewed in (Thiam et al., 2013)). This allows for enlargement of the droplet and perhaps exchange of materials between organelles. An important line of investigation would therefore ask whether lipin-1 is critical for lipid droplet formation in response to mitochondrial stress. It has already been shown that lipin-1 regulates lipid droplets in a variety of tissues (Bou Khalil et al., 2009; Dwyer et al., 2012; Sembongi et al., 2013).

MAM is also critical in neurodegenerative disease. For example, α-synuclein and Alzheimer’s pathogenesis increase MAM (reviewed in (Vance, 2014)). This observation is surprising as loss of pro-fusion factor Mfn2, the main tether between ER and mitochondria, leads to neuropathy (Züchner et al., 2004). Also of interest is the observation that α-synuclein binds lipid droplets (Outeiro and Lindquist, 2003) and Lewy bodies, the pathological accumulation of α-synuclein aggregates, are positive for neuromelanin, a pigment containing lipid droplets (reviewed in (Zucca et al., 2014). We would speculate that perhaps mitochondria stress-induced lipid droplets could act as a quarantine mechanism for α-synuclein aggregates and that maybe this system becomes overwhelmed and forms larger inclusions, Lewy bodies, as regular stress-responses cannot keep up. If so, then pharmacological modulation of lipid droplet dynamics might have beneficial or preventative effects for Parkinson’s pathogenesis.

In sum, we illustrate that HDAC6 and Parkin both play significant roles in lipid homeostasis and quality control pathways. Understanding the overlap between metabolism and neurodegeneration will provide invaluable insights for potential nutrient-based treatments or preventative measures in aging and neurodegeneration.
References


Biography

Meghan Danielle Kapur was born in Rolla, Missouri to Pat and Steve Woods. She grew up with her brother, Jacob, in a variety of Midwestern cities before landing in West Chester, PA. After high school, Meghan attended the University of Delaware where she earned a B.S. in Biochemistry and a minor in Spanish. This was an Honors Degree with Distinction for her participation in the UD Honors Program and for successfully defending a senior thesis. Upon graduation, she was awarded the Questia Drake Award for Women in Science. Midway through her graduate career at Duke University, she married her college sweetheart, Amit Kapur, and was formally welcomed by Madhu, Vijay, and Vima Kapur into the family. She is completing her PhD work in the areas of metabolism and neurodegeneration in Duke University’s Pharmacology program. One of her best accomplishments to date is adopting a rescue dog, Nina.