Nampt-Mediated Nad+ Homeostasis in Skeletal Muscle: Implications for Healthy Aging

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Nampt-Mediated Nad+ Homeostasis in Skeletal Muscle: Implications for Healthy Aging

Abstract

Mammalian skeletal muscle is a highly dynamic organ capable of structural and metabolic remodeling in response to exercise demands, nutrient supply, and environmental insults. Muscle also plays a central role in the maintenance of whole-body energy balance, capable of both storing and oxidizing carbohydrate and lipid fuels. The course of natural aging leads to a gradual decline in the mass, strength, and oxidative capacity of skeletal muscle, which increases the susceptibility of the elderly to frailty and metabolic diseases, such as Type II Diabetes. Ectopic muscle lipids can also exacerbate the metabolic complications of obesity, prompting interest in new means of combating this effect by stimulating aerobic muscle metabolism. Several lines of evidence have converged on the fundamental electron-shuttling metabolite, nicotinamide adenine dinucleotide (NAD), as a co-factor and signaling intermediate uniquely positioned to modulate whole-body energy balance through transcriptional and post-translational mechanisms. Though it has long been known that NAD supports essential metabolic pathways in muscle, attempts to stimulate oxidative metabolism by systemically increasing intracellular NAD concentration have been unable to resolve the specific contribution, if any, of skeletal muscle to the resulting phenotypes. Furthermore, the observation that muscle NAD content tends to decline with age has never been demonstrated to have functional consequences. I have addressed these questions experimentally by generating transgenic mice with altered muscle expression of nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme in the mammalian NAD salvage biosynthetic pathway. Despite effectively increasing the muscle NAD pool, Nampt expression alone is not sufficient to stimulate mitochondrial function in young mice and provides no protection from diet-induced obesity. However, aging these mice for 24 months revealed partial preservation of youthful exercise capacity through primarily non-transcriptional mechanisms. Specific deletion of Nampt in adult mice elucidated a critical threshold of NAD required to maintain the exercise performance, as well as the mass and strength of muscle, by directly supporting aerobic ATP synthesis. Importantly, these parameters can be rapidly and uniformly restored by administration of the NAD precursor, nicotinamide riboside. These studies indicate that loss of NAD homeostasis is a reversible cause of skeletal muscle dysfunction with wide-ranging therapeutic implications.

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First Advisor

Joseph A. Baur

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Keywords
metabolism, muscle, NAD, NAMPT, nicotinamide riboside, sarcopenia

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NAMPT-MEDIATED NAD⁺ HOMEOSTASIS IN SKELETAL MUSCLE:
IMPLICATIONS FOR HEALTHY AGING

David W. Frederick

A DISSERTATION
in
Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania
in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

2016

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NAMPT-MEDIATED NAD⁺ HOMEOSTASIS IN SKELETAL MUSCLE: IMPLICATIONS FOR HEALTHY AGING

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David William Frederick

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For my parents, Dr. William G. and Maryann G. Frederick
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ABSTRACT

NAMPT-MEDIATED NAD⁺ HOMEOSTASIS IN SKELETAL MUSCLE:
IMPLICATIONS FOR HEALTHY AGING

David W. Frederick
Joseph A. Baur, Ph.D.

Mammalian skeletal muscle is a highly dynamic organ capable of structural and
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insults. Muscle also plays a central role in the maintenance of whole-body energy balance,
capable of both storing and oxidizing carbohydrate and lipid fuels. The course of natural aging
leads to a gradual decline in the mass, strength, and oxidative capacity of skeletal muscle, which
increases the susceptibility of the elderly to frailty and metabolic diseases, such as Type II
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prompting interest in new means of combating this effect by stimulating aerobic muscle
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metabolite, nicotinamide adenine dinucleotide (NAD), as a co-factor and signaling intermediate
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acac</td>
<td>Acetoacetate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP kinase</td>
</tr>
<tr>
<td>AntA or AnA</td>
<td>Antimycin A</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BHB</td>
<td>Beta-hydroxybutyrate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Ckmm</td>
<td>Muscle creatine kinase M</td>
</tr>
<tr>
<td>CR</td>
<td>Calorie restriction</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
</tr>
<tr>
<td>CyC</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus muscle</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>Mal</td>
<td>Malate</td>
</tr>
<tr>
<td>MART</td>
<td>Mono-ADP ribosyltransferase</td>
</tr>
<tr>
<td>MIM</td>
<td>Metabolite indicator method</td>
</tr>
<tr>
<td>mNKO</td>
<td>Skeletal-muscle specific Nampt Knockout mouse line</td>
</tr>
<tr>
<td>mNTG or NNC</td>
<td>Muscle-specific Nampt transgenic mouse line (homozygous)</td>
</tr>
<tr>
<td>mNTG(het) or NC</td>
<td>Muscle-specific Nampt transgenic mouse line (heterozygous)</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>mRNA-seq</td>
<td>Messenger RNA sequencing</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAD* or NAD</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADGH</td>
<td>NAD glycohydrolase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>Nampt</td>
<td>Nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>NMN</td>
<td>Nicotinamide mononucleotide</td>
</tr>
<tr>
<td>NMNAT</td>
<td>Nicotinamide mononucleotide adenyltransferase</td>
</tr>
<tr>
<td>NNMT</td>
<td>Nicotinamide N-methyl transferase</td>
</tr>
<tr>
<td>NR</td>
<td>Nicotinamide riboside</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP ribose polymerase</td>
</tr>
<tr>
<td>PGC-1a</td>
<td>Peroxisome proliferator-activated receptor gamma, co-activator 1 alpha</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PRPP</td>
<td>Phosphoribosyl pyrophosphate</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Pyr</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>RC</td>
<td>Regular chow</td>
</tr>
<tr>
<td>Redox</td>
<td>Reduction-oxidation</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rot</td>
<td>Rotenone</td>
</tr>
<tr>
<td>Ryr1</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sir2</td>
<td>Silent information regulator 2</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STAC</td>
<td>Sirtuin-activating compound</td>
</tr>
<tr>
<td>Suc</td>
<td>Succinate</td>
</tr>
<tr>
<td>TB.N</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>TB.Th</td>
<td>Trabecular thickness</td>
</tr>
<tr>
<td>TB.Sp</td>
<td>Trabecular spacing</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume of carbon dioxide produced</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume of oxygen consumed</td>
</tr>
<tr>
<td>VOX BV/TV</td>
<td>Relative bone volume (%)</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION

“It may indeed be considered a success of general physiology and its mode of experimenting, that the chemical dynamics of a highly differentiated organ like the muscle could be partly revealed by the study of alcoholic fermentation of yeast”.

-Otto Fritz Meyerhof
Research Professor of Physiological Chemistry at the University of Pennsylvania and Nobel Laureate in Medicine, 1924
Conventional wisdom holds that aging is an inevitable fact of life. Classically defined, the biological process of aging is a gradual physiological deterioration that results in an increased probability of death over time (Comfort, 1954). It follows that age is a primary risk factor associated with the leading causes of death in the developed world, including cancer, diabetes, and cardiovascular disease (Niccoli and Partridge, 2012). While little can be done to affect the passage of time, the biological mechanisms underlying the aging process are beginning to be understood and manipulated in ways that hold significant promise for human health.

Throughout evolutionary history, the lifespan of an individual human was largely limited by extrinsic and environmental factors, such as predation, famine, and infectious disease. In fact, the average human born in the Neolithic Era was estimated to survive no more than 30 years (Galor and Moav, 2007). However, after millennia of technological and medical progress, a child born in the United States today has an estimated life expectancy of more than 80 years (Niccoli and Partridge, 2012). This staggering improvement over prehistoric standards reflects a shift from stochastic to inherent biological limits of lifespan. Accordingly, modern medicine is increasingly focused on identifying points of potential intervention during the age-related failure of specific organs, including the brain, liver, heart, and skeletal muscle. One such focal point, which has gained considerable attention over the last ten years, is the ubiquitous metabolic intermediary, nicotinamide adenine dinucleotide (NAD⁺ or NAD). This versatile biomolecule exhibits myriad functions at the intersection of intracellular signaling, metabolism, and nutrition. New therapeutics targeting the synthesis of NAD generally aim to lengthen the duration of disease-free survival, or healthspan, if not the lifespan of humans.

As one of the largest organs by mass, essential to locomotion and dynamically responsive to both exercise and nutritional stress, skeletal muscle is a key guardian of healthspan. Equally important to its function in the respiration, feeding, and reproduction of all mammals, skeletal muscle is central to the maintenance of whole body energy balance. Composed primarily of post-mitotic fibers, muscle transports, stores, and oxidizes carbohydrate and lipid fuels to support the energetic demands of contraction, representing the largest sink for
postprandial glucose (Kelley et al., 2002). The natural, age-related loss of muscle mass and contractile strength, termed sarcopenia, is a significant risk factor for fall-related injuries, loss of mobility, and associated metabolic syndromes in the elderly (Marzetti and Leeuwenburgh, 2006). These comorbidities are the source of significant economic costs to the world healthcare system, which are only expected to increase with life expectancy. While the exact causes of sarcopenia remain a matter of debate, there is considerable evidence that mitochondrial dysfunction contributes to the characteristic reduction in both the size and number of muscle fibers (Romanick et al., 2013) and contributes to ectopic lipid accumulation, often resulting in muscle glucose intolerance and development of Type II Diabetes (Johannsen et al., 2011). Thus, approaches to specifically maintain the quantity and function of skeletal muscle mitochondria may be therapeutically useful to an aging population.

A Relationship Between Aging and Mitochondrial Function

What components of the muscle energy-producing machinery lose efficiency over time? To address this question in the most clinically relevant manner, physiologists have relied on classic biochemical analyses of muscle biopsies in combination with stable isotope tracers and advanced imaging modalities, such as nuclear magnetic resonance spectroscopy, to monitor parameters such as tricarboxylic acid cycle (TCA) flux and ATP production rate in the muscles of human patients (Petersen et al., 2003, 2015; Short et al., 2005). A clear negative correlation was identified between the age of a subject and the complex I-dependent mitochondrial ATP production rate, declining approximately 8% per decade of adult life (Short et al., 2005). This trend was coincident with an increase in oxidative damage to deoxyguanosine nucleotides and declines in both the total mtDNA content and protein content of several mitochondrial enzymes, including those of the TCA cycle, such as citrate synthase (Short et al., 2005). At the cellular level, diminished mitochondrial capacity appears to result in the accumulation of intramyocellular
lipids, likely responsible for the observed decreases in postprandial glucose tolerance and muscle insulin sensitivity with age (Petersen et al., 2003; Samuel and Shulman, 2016; Short et al., 2005). Furthermore, by using the relative rates of citrate synthase and pyruvate dehydrogenase as proxies of fatty acid and glucose-fueled TCA cycle activity, respectively, Petersen and colleagues concluded that muscle from healthy elderly subjects exhibits a diminished ability to switch between utilization of lipid and glucose substrates, perhaps driving susceptibility to Type II Diabetes (Petersen et al., 2015). Interestingly, essential turnover of the TCA cycle, as well as the activities of mitochondrial complex I, pyruvate dehydrogenase, and 3-hydroxy-acyl-CoA dehydrogenase require the oxidation and reduction of NAD co-factors, while signaling through NAD-dependent transcription has been proposed to mediate fuel selection in muscle fibers (Gerhart-Hines et al., 2007). Together, these studies hint that jeopardizing specific points of metabolic control in muscle, some of which require NAD, can eventually lead to more dire consequences for the energy balance of the organ and organism as a whole.

While several poorly understood mechanisms are likely responsible for the age-related decline in mitochondrial function, one unifying theory relating to oxidative stress dominates modern gerontology. The tendency of molecular oxygen to spontaneously react with macromolecules over time is potentially detrimental to the finely tuned kinetics of enzymes and the information encoded in nucleic acids, creating a scenario in which a decline in metabolic efficiency can become self-reinforcing. This idea forms the basis of the mitochondrial free radical theory of aging, first described by Harman in the 1950s (Harman, 1956) and highly credible to this day. According to this theory, the flow of electrons through aerobic metabolism inevitably generates byproducts in the form of oxygen radicals, a subset of so-called reactive oxygen species (ROS), which can damage the structures of biological macromolecules in close proximity by stripping away electrons. Post-mitotic tissues, such as neurons and muscle, would be expected to be particularly susceptible to the accumulation of such damage, since cellular turnover is so slow. It is now appreciated that eukaryotic cells defend against these superoxide
and hydroxyl anions by expressing antioxidant enzymes, such as superoxide dismutase and catalase, detoxifying the radicals back into molecular oxygen and water (Schriner, 2005).

Despite the aforementioned correlative evidence in support of Harman’s theory, it was several decades before experimental validation in a mammalian system was feasible. In 2005, the Rabinovitch group generated a transgenic mouse line overexpressing a catalase enzyme specifically targeted to mitochondria. So-called MCat mice clearly exhibited fewer age-related markers of oxidative damage, including 8-hydroxyguanosine accumulation in DNA, and an extension of both median and maximal lifespan (Schriner, 2005). Several subsequent studies elucidated the nature of the protective effects of ROS scavenging in MCat muscle. The Marks group demonstrated improved exercise capacity in relatively elderly 24-month-old MCat mice compared to age-matched controls, and observed a similar pattern in the strength of isolated muscles and the maximal calcium load of the sarcoplasmic reticulum (SR) (Umanskaya et al., 2014). They attributed the latter effect to diminished oxidation of the aged MCat muscle ryanodine receptors (RyR1), which gate the efflux of calcium from the SR and were previously shown to contribute to age-related muscle weakness by leaking ions after oxidative damage (Umanskaya et al., 2014). Importantly, another study from the Shulman group indicated that MCat mice had a superior ability to maintain muscle mitochondrial function and ATP synthesis into old age, and were further protected from lipid-induced muscle insulin resistance (Lee et al., 2010). Taken together, these studies indicate that antioxidant defenses can retard the deterioration of mitochondrial function in muscle to maintain both the local contractile apparatus and whole body energy homeostasis.

If the process of aging is even partly dictated by mitochondrial integrity, one might expect specific mitochondrial insults to accelerate the development of age-related phenotypes. Concurrent with investigations of the MCat mouse, researchers in the laboratory of N.-G. Larsson generated another transgenic mouse line in order to test this idea directly. The so-called PolG line was designed to carry a germline mutation in the nuclear gene encoding the proofreading domain of the polymerase responsible for replicating mitochondrial DNA (mtDNA), allowing spontaneous
errors in mtDNA replication to go uncorrected. PolG mice appeared normal for the first eight months of life, despite a significantly increased mtDNA mutational load, but soon developed clear signs of premature aging including greying, hair loss, kyphosis, and maximal survival only about half that of wildtype littersmates (Trifunovic et al., 2004). Since mtDNA encodes critical components of the electron transport complexes, this study seemed to validate the inverse relationship between mitochondrial integrity and the rate of aging. Importantly, a subsequent study using an independently generated PolG line with the same mutation did not find evidence of increased ROS generation in mitochondria isolated from PolG heart or liver. Rather, mutant mice exhibited an increase in the age-related appearance of cleaved caspase-3, a marker of apoptosis, in several tissues, including muscle (Kujoth, 2005). Accordingly, PolG mice exhibit accelerated development of sarcopenia, losing approximately 10% of hindlimb muscle mass by the relatively young age of 9 months. Thus, cell death initiated by mitochondrial dysfunction may play a role in the deterioration of muscle and other tissues with age.

Mitochondria are capable of regulating aged muscle cell death through programmed and nonprogrammed mechanisms. Upon insult, regulated release of specific factors from the mitochondrial matrix, including cytochrome C, Apoptosis Inducing Factor, and Endonuclease G, triggers an orchestrated caspase-dependent signaling cascade leading to DNA fragmentation, proteolysis, and cell death by apoptosis (Marzetti and Leeuwenburgh, 2006). However, this process is relatively energy-intensive. In cases where mitochondrial function is crippled and ATP severely depleted, a cell may succumb to unregulated death by necrosis, rendering cell membranes permeable and affected tissue regions identifiable by uptake of vital dyes, such as Evans blue (Hamer et al., 2002; Leist et al., 1997). While a combination of apoptosis and necrosis likely contributes to most muscle pathologies, including sarcopenia, shared mitochondrial mechanisms might be involved in regulating both routes of cell death. One example is the mitochondrial permeability transition pore (MPTP), which represents a primitive self-destruct switch when forced open by excessive ROS, calcium load, energetic stress, or signaling events (Wallace and Fan, 2009). As such, mice deficient for the stress-responsive MPTP activator,
cyclophilin D, or treated with a pharmacological cyclophilin inhibitor exhibited reduced myonecrosis in several distinct mouse models of innate muscular dystrophy (Millay et al., 2008). Similarly, cardiomyocytes containing insufficient levels of oxidized NAD in the mitochondria are susceptible to stress-induced cell death through MPTP opening (Karamanlidis et al., 2013). This suggests that the MPTP is a critical node through which NAD dynamics might influence cell death. Accordingly, methods of maintaining tissue NAD levels may help to avoid this fate.

**Aging as a Plastic Biological Process**

The view that aging is a simple consequence of thermodynamics has yielded in recent decades to the appreciation that aging is at least partially a regulated biological process. This paradigm shift began in 1935, when McKay, Crowell, and Maynard reported that reducing caloric intake was sufficient to extend the lifespan of rats (McCay et al., 1935). Since that time, it has been confirmed that calorie restriction (CR), in the absence of malnutrition, extends the mean and maximal survival of eukaryotes as diverse as yeast and worms to flies and rodents by as much as 40% (Weindruch, 1988), with conflicting but promising data from primates (Colman et al., 2009; Mattison et al., 2012). Perhaps more importantly, CR uniformly delays the development of age-related phenotypes in numerous species. In mammals, these benefits translate to decreased incidence or delayed onset of cancer, cardiovascular disease, and diabetes (Colman et al., 2009). The impact of CR on skeletal muscle is equally profound. Reducing the calorie intake of non-human primates by 30% over 17 years yielded a much slower decline in weight-adjusted muscle mass with age, indicating attenuation of sarcopenia (Colman et al., 2008). Importantly, CR limits the production of superoxide radicals and the associated oxidative damage to protein and lipids in rodent skeletal muscle, correlating with complete prevention of the age-related loss of muscle oxidative capacity during lifelong regimens (Baker et al., 2006; Lass et al., 1998). Given these remarkable outcomes, understanding the mechanisms underlying the response to CR in muscle and other tissues offers immense potential for developing therapies to improve healthspan.
The signaling-mediated model of lifespan regulation is not intuitive. Yet, there is now overwhelming evidence that the physiological response to CR depends on metabolic reprogramming, coupling nutrient availability, energy charge, and intracellular reduction-oxidation (redox) state to the regulation of transcription and post-translational modifications (PTMs). Some of the earliest data in support of this model was obtained in a diverse array of eukaryotic model systems by identifying spontaneous single gene mutations in long-lived strains, such as *daf-2* in worms, *methuselah* in flies, and *p66sch* in mice (Guarente and Kenyon, 2000). Further manipulations of genetically tractable lower eukaryotes have determined that, in addition to antioxidant mediators, longevity-promoting programs typically converge on just three highly conserved signaling pathways, in which insulin/insulin-like growth factor (IGF), mammalian target of rapamycin (mTOR), and sirtuins are central players (Scott and DeFrancesco, 2015), forming a short list of targets for potential anti-aging interventions.

Pathways including IGF, mTOR, and sirtuins appear to connect environmental cues to physiological responses via complementary and non-redundant functions. For example, the insulin and insulin-like growth factors circulate between cells in vertebrates to signal nutrient availability and stimulate energy metabolism or growth of target cells by binding to specific receptor tyrosine kinases (Lemmon and Schlessinger, 2010). In 2003, it was reported that heterozygous mutations in the mouse IGF receptor, homologous to those already established to extend the lifespan of flies and worms, were indeed sufficient to extend mammalian survival by 26% (Holzenberger et al., 2003). Downstream of the insulin family of growth factor receptors, mTOR is a serine/threonine kinase and signaling nexus that receives additional inputs from amino acid levels to control the balance between protein synthesis and autophagy. As its name implies, mTOR is potently inhibited by rapamycin- an effect observed to extend the lifespan of yeast, worms, and flies (Laplante and Sabatini, 2012). In 2009, Harrison and colleagues confirmed the speculation that mice, too, benefit from rapamycin treatment, surviving 9-14% longer when the compound was supplemented in the diet, even beginning at the advanced age of 600 days (Harrison et al., 2009). Finally, mammalian sirtuins, orthologues of the yeast Sir2, are a
class of histone deacetylase (HDAC) affecting the acetylation state of over 100 intracellular
targets, including histones and transcription factors (Nakagawa and Guarente, 2014). The closest
mammalian homologue, Sirt1, is localized to both the cytosol and nucleus and appears to be
required for CR-mediated lifespan extension in mice, though caveats abound (Li et al., 2008;
Mercken et al., 2013). Interest in the activity and regulation of sirtuins has uncovered novel
connections between nutrient restriction and cell metabolism, as described below. Importantly,
the combination of these observations indicates that the CR response is at least partially
mediated by chromatin dynamics.

Beginning even before the discovery of mammalian sirtuins, the joint work of Weindruch
and Prolla was the first to utilize DNA microarrays and other high throughput techniques in
attempts to isolate the physiological signature of CR in different tissues. In skeletal muscle, their
team found that aging a standard C57BL/6 mouse line from 5 to 30 months was associated with
significant increases in the expression of genes responding to oxidative and genotoxic stress and
decreases in genes related to glycolysis and mitochondrial function (Lee et al., 1999).
Importantly, lifelong restriction of caloric intake to 76% of ad libitum levels attenuated a majority of
these changes, regardless of the directionality (Lee et al., 1999). Subsequent work using
messenger RNA sequencing (mRNAseq) to allow deeper coverage of the muscle transcriptome,
as well as the identification of mRNA splice variants, has confirmed these major findings and
additionally noted CR-mediated suppression of genes related to the actin cytoskeleton and
regulators of muscle development (Dhahbi et al., 2012). Interestingly, the most conserved
transcriptional feature of aging in worms, flies, mice, and humans, appears to be a general
decrease in the expression of nuclear-encoded components of the electron transport chain
(ETC), perhaps as a simple means of limiting free radical production (Zahn et al., 2007).
However, identification of sensitive and specific transcriptional biomarkers of aging in different
species has been less fruitful. A leading candidate in mice is the tumor suppressor p16ink4a,
which is increasingly transcribed during advanced age in almost all tissues, through mechanisms
that can be suppressed by CR (Krishnamurthy et al., 2004). By employing a clever transgenic
approach to selectively induce apoptosis in the p16ink4a-expressing cells of a progeroid mouse model, Baker and colleagues found an increase in the mean fiber diameter of skeletal muscle, which coincided with increases in the time, distance, and work performed in treadmill exercise tests (Baker et al., 2011). Collectively, these experiments demonstrated that it is conceptually feasible to attenuate the age-related decline in the performance of muscle and that altered expression of specific genes may promote the benefits of restricted calorie intake.

In order to rapidly respond to environmental cues, the fundamental energy producing processes of the cell cannot rely on gene expression alone. The advent of high-throughput proteomics has allowed the functional interrogation of previously unappreciated protein PTMs sensitive to the nutritional status of the cell. Among these, lysine acetylation is a reversible PTM which has been detected on over 2,000 different mammalian proteins and is an excellent candidate for regulation of cell metabolism (Guan and Xiong, 2011). Metabolic regulatory enzymes are highly represented in the acetylated peptide pool, including nearly all enzymes in pathways of glycolysis, the TCA cycle, fatty acid oxidation, and glycogen storage (Guan and Xiong, 2011). A survey of the human skeletal muscle proteome identified 2,811 lysine acetylation sites on 941 distinct proteins, including all four core histones, with specific enrichment of peptides related to electron transport and muscle contraction (Lundby et al., 2012). These unbiased studies raise the prospect of what might be described as an acetylation rheostat to modify metabolic flux (Auwerx and Menzies, 2013).

Early studies connecting nutrient stress to lysine acetylation focused primarily on the modification of key transcription factors and transcriptional co-activators. Among these were the tumor suppressor, p53, pro-inflammatory p65, stress-resistant FOXO1, and the mitochondrial regulator, PGC-1a (Zhang and Kraus, 2010). The latter co-activator is especially capable of affecting skeletal muscle remodeling by promoting a shift toward oxidative type I fibers and an increase in the oxidation of fatty acids through transcription of related genes, such as MCAD and CPT-1b (Gerhart-Hines et al., 2007; Lin et al., 2002). Importantly, PGC-1a contains 13 acetylated lysine residues which, when mutated, can ablate the activity of the co-repressor in cultured
Together, these findings highlight the diversity of potential routes for acetylome dynamics to impact the energy balance of muscle and other tissues.

How might dynamic transcriptomes and acetylomes be connected to nutrition? There is accumulating evidence that CR evokes rapid metabolic reprogramming in eukaryotes and that histones are dynamically acetylated, suggesting that epigenetic mechanisms are involved. It is now appreciated that covalent modifications to DNA, including cytosine methylation, as well as the methylation, phosphorylation, and ubiquitination of histone tails, coordinately regulate the accessibility of chromatin to transcriptional machinery in a manner that often relies on intermediates of cellular metabolism (Fan et al., 2015). More importantly, these metabolites fluctuate in response to changes in nutrient availability. For example, the canonical energy sensor AMP kinase (AMPK) has been found to modulate transcription upon ATP depletion by phosphorylating histone 2B, while the histone demethylase activity of Jumonji-C domain containing enzymes requires the TCA cycle intermediate alpha-ketoglutarate (Bungard et al., 2010; Tsukada et al., 2005). Similarly, the addition of acetyl groups to lysine residues by lysine acetyltransferase enzymes relies on a supply of acetyl-CoA, the classical entry vehicle for carbon into the TCA cycle, which can fluctuate in concentration by approximately 10-fold in response to nutritional status (Kaelin and McKnight, 2013). The reverse reaction is catalyzed on these and non-histone substrates by so-called histone deacylase (HDAC) enzymes, generally regulated by protein-protein interactions, phosphorylation, and subcellular localization (Sengupta and Seto, 2004). However, class III HDACs, more commonly known as sirtuins, utilize a unique deacetylation mechanism which requires NAD, an essential electron-shuttling molecule in many metabolic processes. Exactly how deacetylation of specific targets may complete the logic loop connecting CR to metabolic homeostasis is an active area of investigation in the aging field.
The essential functions of NAD in carbohydrate metabolism were discovered in the first half of the twentieth century using the brewer’s yeast, *Saccharomyces cerevisiae*. The existence of NAD and a clue to its biochemical significance was originally deduced by Harden and Young in 1906, who determined that a heat-stable and dialyzable factor in yeast extracts was necessary for the fermentation of sugar into alcohol (Harden and Young, 1906). As early as 1924, Meyerhof recognized that an analogous anaerobic pathway required the same factor to produce lactate in the contracting skeletal muscle of frogs (Otto Meyerhof, 1924). By 1959, Harden, Meyerhof, and three other pioneering biochemists had gone on to win Nobel Prizes for work which informed the structure, synthesis, and chemical reduction of the dinucleotide, after which progress on the study of NAD metabolism slowed for decades (Berger and Ziegler, 2004). Nonetheless, the metabolic processes found to utilize NAD, including glycolysis and oxidative respiration, appeared so fundamental that at least some functions of the metabolite were clearly transferable from single-celled organisms to vertebrates.

It was not until the turn of the millennium that a flurry of activity in the field of yeast genetics identified a novel aspect of NAD biology that might mediate both epigenetics and aging. Leading the field, the Guarente laboratory showed that expression of additional copies of a chromatin-modifying enzyme in yeast, named silent information regulator 2 (Sir2), as well as expression of its closest homologue in *Caenorhabditis elegans*, effectively extended the mean lifespan of both model organisms beyond that of respective wildtype controls (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001). Following hints from pyridine nucleotide-dependent pathways in bacteria, it was discovered that Sir2 incubated with $^{32}$P-NAD could transfer the radioactive phosphate to albumin protein, and that the core catalytic domain responsible was present in at least five mammalian homologues expressed in mouse tissues, including skeletal muscle (Frye, 1999). Months later, this insight was extended to show that Sir2 could more readily
catalyze the reverse reaction, transferring an acetyl group from a lysine residue of histone 4 onto NAD in a novel deacetylation mechanism (Imai et al., 2000). This observation was hailed as a feasible mechanism through which eukaryotes might couple metabolism to epigenetics, effectively adapting to nutrient deprivation by silencing regions of chromatin. It was soon confirmed that NAD was essential to the Sir2-dependent mechanism responsible for extending yeast lifespan during CR (Lin and Guarente, 2000).

Studies of Sir2 enzymology soon shifted focus from the ubiquitous NAD substrate to other metabolites that might serve as intrinsic regulators of catalytic activity. Guarente’s team reasoned that NADH, the reduced form of NAD, might competitively inhibit sirtuins when metabolic flux was high, forming a nutrient-dependent, redox-sensitive feedback mechanism. The finding that 50-300 micomolar concentrations of NADH does inhibit sirtuin activity seems to support this hypothesis (Lin et al., 2004). Others focused on the byproducts of the sirtuin deacetylation reaction that are liberated from NAD, including nicotinamide (NAM) and 2-O-acetyl-ADP ribose, which might exert feedback on the reaction in a non-competitive manner. A stress-responsive yeast nicotinamidase, named Pnc1, did indeed modulate the activity of Sir2 by clearing NAM, independent of steady-state NAD levels (Anderson et al., 2003; Gallo et al., 2004). Impressively, overexpression of Pnc1 was sufficient to extend replicative lifespan to the same extent as CR, hinting that NAD biosynthetic pathways could be intentionally manipulated to control cellular processes which evolved to be NAD-sensitive (Anderson et al., 2002).

Today, it is clear that sirtuins play innumerable roles in the regulation of mammalian metabolism. Most intriguingly, the seven mammalian sirtuin isoforms with homology to Sir2 appear well positioned to suppress the proposed effectors of the free radical theory of aging. Since it is energetically costly to regenerate the NAD consumed by the sirtuin reaction, especially compared to the spontaneous activity of class I, II, and IV HDACs, it seemed plausible that the associated substrates were of fundamental importance to metabolic regulation. Like yeast, deletion of certain sirtuin isoforms (Sirt1, Sirt6, and Sirt7) shortens the lifespan of mice- an observation that has confounded interpretation of the aforementioned finding that CR does not
extend the survival of Sirt1-deficient mice (Li et al., 2008; Mercken et al., 2013). In calorie-restricted muscle, the observed increase in insulin sensitivity has been suggested to result from Sirt1-mediated mitochondrial biogenesis and a switch to fatty acid oxidation following deacetylation of PGC-1a (Gerhart-Hines et al., 2007; Schenk et al., 2011). Sirt6 acts as a tumor suppressor, critical to genome stability and survival, as well as a modulator of inflammation and anaerobic metabolism through interactions with NF-κB and Hif-1α transcription factors, respectively (Kawahara et al., 2009; Mostoslavsky et al., 2006; Zhong et al., 2010). The mitochondrial localization of Sirt3 allows it to deacetylate enzymes of the TCA cycle, such as isocitrate dehydrogenase 2, regulators of oxidative stress, including superoxide dismutase 2, and components of the ETC itself, including those of complexes I, II, and V (Ahn et al., 2008; Qiu et al., 2010; Someya et al., 2010; Vassilopoulos et al., 2014). However, unlike yeast, sirtuin gain-of-function experiments have yielded remarkably mild impacts on longevity. One study found that careful standardization of genetic backgrounds abolished the foundational connection between Sir2 expression and longevity in *C. elegans* and *D. melanogaster* (Burnett et al., 2011). Only in the case of Sirt6, in which overexpression extended median and maximal lifespan of male mice by about 15%, has any effect been observed (Kanfi et al., 2012). Confounding interpretation of this result as an anti-aging intervention, the tumor suppressing nature of Sirt6 is a critical caveat in a model system that often dies of cancer. Mounting evidence suggests that sirtuins are less dominant in the regulation of mouse physiology than originally anticipated.

Though the biology of aging is faster to observe and easier to manipulate in single-celled systems, it is becoming increasingly clear that yeast may be poor surrogates for higher eukaryotes with trillions of cells, complex organ systems, and multi-decade lifespans. For example, the seven mammalian sirtuins each have distinct subcellular localization, substrates, binding partners, and kinetics not found in yeast (Haigis and Sinclair, 2010). While deacetylation is the only NAD-degrading enzymatic reaction in yeast, mammals can perform this function via dozens of mono- or poly-ADP-ribose polymerases (MARTs and PARPs) and NAD glycohydrolase enzymes (NADGHs)(Berger and Ziegler, 2004). Additionally, the decoding of the mammalian
genome identified no enzymes that breakdown NAM to nicotinic acid and ammonia in a manner analogous to Pnc1. Thus, the difficulty in translating sirtuin biology from yeast to mice may stem from the faulty assumption that NAD-dependent signaling is fundamentally similar in these species. Nonetheless, nearly every mechanism proposed to contribute to mammalian aging contains at least one NAD-dependent component, creating an abundance of alternative avenues through which NAD may be manipulated to support mitochondrial function and healthy aging.

The Diverse Roles of NAD in Mammalian Tissues

The conserved presence of NAD in all living cells is a testament to the omnipresence of the molecule throughout six billion years of evolution. Perhaps not surprisingly, recent research into NAD and NADP-binding domains of human proteins has identified these features in greater than 5% of the established proteome (Hua et al., 2014). The NAD-dependence of sirtuins highlights the atypical nature of the dinucleotide as both a redox cofactor and a co-substrate, sparking a resurgence of interest in the biology of NAD homeostasis. Of course, the most fundamental and well-studied role of NAD in all domains of life is to facilitate the stepwise oxidation of metabolic fuels for ATP production. When considering the consequences of fluctuations in NAD homeostasis, one must consider the NAD-dependent steps in glycolysis and OXPHOS.

When utilizing carbohydrates for fuel, the reduction of NAD to NADH is required to convert glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in a reaction essential to glycolysis, mediated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For glucose-derived carbon to leave the cell as lactate or enter the TCA cycle as acetyl-CoA, NADH must be oxidized back to NAD through lactate dehydrogenase or pyruvate dehydrogenase, respectively. This relationship between lactate and pyruvate, especially, makes the ratio of the two metabolites a useful indicator of the overall redox state of NAD in the cytosol. Without sufficient access to NAD, defined by the $K_m$ of ~60 µM in muscle, glycolytic flux through GAPDH would be abolished.
(Lambeir et al., 1991). Instead, upstream metabolites might be forced through alternative routes, such as the pentose phosphate pathway, typically responsible for maintaining the NADP/NADPH redox couple and shunting carbons into nucleotide synthesis. NADP is derived from phosphorylation of NAD and provides reducing power to glutathione proteins in order to buffer reactive oxygen species in the cytosol (Lass et al., 1998). These points of NAD-dependence in the cytosol allow ATP synthesis by phosphoglycerate kinase and pyruvate kinase, as well as antioxidant protection, independent of mitochondrial function.

One of the essential functions of NAD as a co-factor is to help generate and maintain mitochondrial membrane potential by serving as a key intermediary between the TCA cycle and the ETC. Additionally, within mitochondria, pyruvate dehydrogenase and two dehydrogenases of the TCA cycle (isocitrate dehydrogenase and alpha ketoglutarate dehydrogenase) are so-named because they transfer a proton from their respective substrates to an NAD cofactor, which becomes re-oxidized upon interaction with ETC complex I. NAD-consuming enzymes in the matrix are limited to Sirt3, Sirt 4, and Sirt5, and are collectively believed to play only subtle roles in mitochondrial substrate selection and ammonia detoxification (Haigis and Sinclair, 2010). As such, deletion of Sirt3 in liver and muscle is reportedly inconsequential to whole body metabolic homeostasis (Fernandez-Marcos et al., 2012). Nonetheless, the presence of NAD-consuming enzymes in the matrix creates demand for import or local synthesis of the dinucleotide; both matters of ongoing investigation. However, it is clear that the mitochondrial NAD pool is distinct from that of the nucleus and cytosol and may be independently regulated to provide cells with a survival advantage during nutritional or genotoxic stress (Yang et al., 2007a).

In the cytosol of mammalian cells, a wide range of enzymes consume NAD and liberate NAM, including PARPs, MARTs, and NADGHs. PARPs, the most commonly studied family of NAD-consumers, form branching polymers of ADP-ribose on histones near sites of DNA damage as an initiating step in the repair process. While the structure of chromatin-associated PARP polymers has been known since the 1960s, it was not until 1983 that the NAD-dependence of PARPs was identified as a point of metabolic susceptibility, such that hyperactivation of PARP is
capable of restricting ATP production to the point of necrotic cell death (Kraus, 2015). The survival of rapidly dividing cells can also be jeopardized by preventing the repair of DNA damage, forming the logic behind PARP inhibitors as a class of anti-cancer drugs. An alternative and less common modulator of protein function results from the transfer of single ADP and ribose moieties of an NAD molecule to a protein substrate in a mono-ADP ribosylation catalyzed by MARTs. An example of this activity is found on the extracellular matrix protein integrin alpha-7, which is ADP ribosylated to increase its affinity for the muscle-specific ligand laminin, perhaps affecting the structural integrity of skeletal muscle (Zhao et al., 2005). Finally, NADGHs convert the dinucleotide into cyclic ADP-ribose and NAM, the former of which serves as a potent calcium-mobilizing metabolite upon interaction with ion channels in the sarcomeric reticulum of muscle (Berger and Ziegler, 2004). The ecto-enzyme CD38 is one such highly active NADGH, which facilitates agonist-induced elevation of cytosolic calcium (Malavasi et al., 2008). The degree to which these enzymes compete for a limited substrate pool is a point of active speculation, as those with the lowest effective $K_m$ for NAD would be expected to take priority. Regardless of this competition, the loss of redox-active NAD through PARP, MART, and NADGH-mediated catalysis creates a critical need for local regeneration of the dinucleotide in order to maintain cellular energetics.

**Targeting NAD for Therapeutic Indications**

The discovery that sirtuins may mediate the physiological benefits of CR raised the exciting prospect that related signaling pathways might be stimulated to reproduce these benefits without the need for dietary modification. The development of so-called CR-mimetics has ensued with moderately successful results in lower organisms. Among these compounds, the naturally occurring polyphenol resveratrol was found to prevent early mortality associated with diet-induced obesity, but failed to extend the lifespan of otherwise healthy mice (Baur et al., 2006). SRT1720, a rationally designed sirtuin-activating compound (STAC), was found to offer similar protection in
the context of high fat diet, effectively doubling the treadmill running distance of obese mice (Feige et al., 2008). Another STAC, SRT2104, was reported to benefit the mean survival time of mice on a standard diet and partially protect against the loss of bone and muscle mass during hindlimb suspension (Mercken et al., 2014). While it appeared that STACs could influence mouse physiology under the right conditions, their efficacy in preventing common age-related diseases was underwhelming, and further dogged by evidence that the compounds exerted myriad off-target effects and might not be true Sirt1 activators (Pacholec et al., 2010). Despite evidence that glutamic acid 230 is required for in vitro activation of Sirt1 by STACs, implying a direct allosteric interaction, there remains no evidence that the compounds act by increasing the affinity of the enzyme for either the acetylated substrate or NAD (Hubbard et al., 2013). If the CR response is even partly mediated by sirtuins, a simpler alternative to allosteric activation is the manipulation of NAD metabolism itself.

The study of NAD biology was undoubtedly revived by the excitement surrounding the discovery of sirtuins. Central to the purported regulation of sirtuin activity in vivo is the enhanced availability of NAD under conditions of CR (Guarente, 2011). However, the field was founded on remarkably inconsistent data over whether CR transiently decreased the reduction of NAD to NADH through metabolic flux, increased the chemical conversion and clearance of NAM, increased the activity of NAD biosynthetic pathways, or somehow combined these possibilities. The school of thought, led by the Guarente lab, that CR increases the NAD/NADH redox ratio in numerous tissues to stimulate NAD-dependent processes has gained favor in recent years, though careful observers have pointed out classical studies that argue to the contrary (Chen et al., 2008; Kaelin and McKnight, 2013). Perhaps due to the technical challenges associated with measuring tissue and organelle-specific redox states under various dietary conditions, these issues remain largely unresolved.

In order to circumvent the gaps in understanding surrounding pyridine nucleotide metabolism in mammals, several groups attempted to manipulate the intracellular NAD pool by selectively deleting prominent NAD-consuming enzymes. Consistent with its role as a ubiquitous
membrane-associated NADase, germline deletion of the NADGH CD38 resulted in the elevation of NAD by up to ten-fold in the brain, liver, heart, lung, kidney, and testis of mice (Aksoy et al., 2006). Though these mice were originally generated to study specific roles of CD38 in immune cells, the impressive gains in tissue NAD levels, including a three-fold increase in muscle, made them the ideal system in which to test whether CR-like phenotypes emerged. Though no metabolic phenotypes were reported under basal conditions, CD38 null mice exhibited a higher metabolic rate than wildtype mice after four weeks of high fat feeding, and subsequently exhibited blunted weight gain, freedom from intrahepatic and intramyocellular lipid accumulation, and maintenance of glucose tolerance (Barbosa et al., 2007). Furthermore, co-administration of the putative Sirt1 inhibitor, sirtinol, in the same study appeared to diminish the body weight protection on high fat diet, implicating NAD-dependent deacetylation of metabolic substrates as a foundation of the phenotype. Following similar logic, the Auwerx laboratory described the metabolic phenotype of a mouse line deficient for PARP1, which was generated over a decade earlier for studies of genome stability (de Murcia et al., 1997). As anticipated, muscle and brown adipose from PARP1 null mice also contained more NAD than that of wildtype mice, contributing to a higher basal metabolic rate and protection from diet-induced obesity (Bai et al., 2011a). In related reports, the same group found that not only did PARP2 deletion produced highly similar phenotypes, but that administration of pharmacological PARP inhibitors to wildtype mice was sufficient to increase treadmill exercise capacity by the driving mitochondrial respiration and citrate synthase activity of skeletal muscle in a Sirt1-dependent manner (Bai et al., 2011b; Pirinen et al., 2014). Though multiple factors could eventually confound the health of animals lacking NADGH or PARP activity, the uniform enhancements of oxidative metabolism appeared to be linked by the common element of elevated NAD.

An alternative and perhaps safer means of stimulating NAD-dependent biological processes is to increase the rate of NAD synthesis. The formation of a dinucleotide from nicotinamide mononucleotide (NMN) and ATP is an essential process in all cells, which must be tightly regulated in order to maintain the redox-sensitive steps of glycolysis and the TCA cycle
after NAD consumption. Accordingly, three main strategies have evolved to maintain the mammalian NAD pool. Starting with the essential amino acid tryptophan, cells in the liver and kidneys can produce NAD *de novo* through an eight-step biosynthetic pathway known as the kynurenine pathway. Nicotinic acid can enter this sequence midway in a three-step synthetic route known as the Preiss-Handler pathway. Together, these means form the deamidated route of NAD synthesis, in which NAD synthase utilizes nicotinic acid adenine dinucleotide in the final step (Mori et al., 2014). However, in most tissues, the existence of an NAD salvage pathway achieves the same end in two steps by recovering the NAM breakdown product of NAD-consuming enzymes and recombining it with the nucleotide precursor, phosphoribosyl pyrophosphate (PRPP), and ATP. Critically, the intermediates in this amidated synthetic pathway can be supplemented through the diet to enhance production of NAD (Mori et al., 2014; Verdin, 2015). Vitamin B3, or niacin, the essential water-soluble mixture of NAM and nicotinic acid, can enter both the Preiss-Handler and NAD salvage pathway through phosphoribosyltransferase enzymes to form the mononucleotide precursor to NAD. When administered in gram quantities to humans, niacin transiently increases hepatic NAD content and circulating levels of high-density lipoprotein, while lowering plasma triglycerides (Sauve, 2007). However, it is likely that the lipid-lowering effects of niacin are mediated by interactions between nicotinic acid and the G-protein coupled receptor GPR109A, more so than by an NAD intermediate, since NAM alone is far less potent at treating dyslipidemia (Cantó et al., 2015). The search for therapeutic alternatives to niacin led to the 2004 discovery that nicotinamide riboside (NR), a trace nutrient found in breast milk, could serve as an NAD precursor by being phosphorylated to NMN via dedicated kinases conserved from yeast to mammals (Figure 1.1) (Bieganowski and Brenner, 2004). Furthermore, the finding that NR appeared to activate Sir2 in yeast and effectively extend the lifespan of wildtype yeast cells suggested that NR may also stimulate the very NAD-dependent processes responsible for the benefits of CR in mammals (Belenky et al., 2007a).

The idea that protecting or enhancing the NAD pool might have therapeutic implications originated in the neurosciences and quickly spread to other subfields of physiology.
Contemporaneous with the explosion of interest in sirtuins, a genetic mutation was characterized in a line of mice known as C57BL/WldS, which appeared protected from the so-called Wallerian degeneration of axons that occurs distal to a site of neuronal injury. The WldS genotype was found to encode a novel fusion protein between ubiquitination factor E4B and nicotinamide mononucleotide adenylyltransferase (NMNAT), the final enzyme of the NAD salvage pathway responsible for the ATP-dependent conversion of NMN to NAD (Mack et al., 2001). The fusion protein was observed to protect damaged neurons in a dose-dependent manner by enhancing the NMNAT catalytic activity, indicating that NAD loss might mediate Wallerian degeneration.

Soon after, hints that NAD precursors might have neuroprotective indications were uncovered, when it was reported that neurons explanted from the dorsal root ganglion of mice survived longer in culture when supplemented with either NR, NMN, or NAD itself (Sasaki et al., 2006). It was soon demonstrated that NR and related amide, ester, and acid nucleoside derivatives could indeed increase the NAD content of three commonly studied human and mouse cell lines by up to 260% without overt toxicity (Yang et al., 2007b). While the specific mechanisms of entry and conversion of these molecules into NAD has never been demonstrated, the prevailing model presumes that cells and tissues expressing at least one of the two NR kinase isoforms can directly take up and phosphorylate NR, allowing it to enter the NAD salvage pathway as NMN. Perhaps due to its intuitive nature, this model remains unchallenged.

Consistent with expectations, dietary supplementation of NR has a profound impact on energy balance in mice. Seminal work from the Auwerx laboratory has shown that NR kinase is highly expressed in muscle, such that a dietary NR dose of 400 mg/kg/day appears to stimulate oxidative metabolism of this tissue, concomitant with mild increases in the NAD content of muscle, liver, and brown adipose (Cantó et al., 2012). Furthermore, the increase in muscle mitochondrial proteins and mtDNA content under these conditions coincided with increased oxygen consumption and treadmill performance when challenged with a high fat diet, resulting in protection from diet-induced obesity in a manner reminiscent of the CD38-deficient mouse line (Barbosa et al., 2007; Cantó et al., 2012). Importantly, recent reports have described the utility of
this NR regimen for reversing the effects of two mitochondrial myopathies in mice, alleviating the
symptoms and delaying the progression of muscle-crippling phenotypes (Cerutti et al., 2014a;
Khan et al., 2014a). These findings are noteworthy because they indicate either that inborn errors
of metabolism can deplete NAD from muscle, or that NAD-dependent processes can be
stimulated to promote mitochondrial quality control.

Other studies have favored NMN, the phosphorylation product of NR kinase, as a more
direct NAD precursor. By injecting NMN directly into mice fed a high fat diet, Yoshino and
colleagues correlated the resulting improvements in glucose tolerance with elevated NAD in liver,
white adipose, and muscle (Yoshino et al., 2011). Importantly, these authors noted that the
natural, age-related loss of glucose tolerance could also be improved with NMN, perhaps by
restoring the observed decline in the NAD content of adipose and muscle in mice aged more than
24 months. This concept was examined further in 22-month-old mice, in which NMN
administration was reported to completely reverse the age-related decline in muscle NAD and
ATP content (Gomes et al., 2013). The treatment also mirrored the enhanced transcription of
mtDNA-encoded genes observed during CR, thought to partially mediate the associated
mitochondrial remodeling. As no limiting side effects have yet been reported, strategies to
enhance NAD synthesis in mammalian tissues may hold the greatest potential for improving the
metabolic fitness of the obese and elderly.

*Defining the Role of Nampt in Muscle Metabolic Homeostasis*

Despite their potent influence on mammalian physiology, NAD precursors are still
administered as mere nutritional supplements of naturally occurring compounds. Reports of the
effects of these compounds have dramatically outpaced understanding of their distribution in the
body. Accordingly, targeted genetic approaches to increase NAD synthesis in specific biological
compartments may be far more useful for determining the sites of NAD action *in vivo*.
The ubiquitous distribution and compact enzymatic sequence of the NAD salvage pathway in mammalian cells offers an ideal avenue for such studies. In this pathway, nicotinamide phosphoribosyltransferase (Nampt) first combines NAM with PRPP to form NMN. The NMN product has no known intracellular fate other than to be reversibly combined with ATP by three isoforms of NMNAT to produce NAD (Figure 1.1)(Berger et al., 2005). Surprisingly, NMNAT1 is the only member of this pathway that has been overexpressed in mouse tissue for purposes of modulating metabolism. By introducing the cDNA into mouse tibialis anterior muscle by electroporation, Gomes and colleagues demonstrated a resulting transient increase in NMNAT1 protein expression and a fourteen-fold increase in transcription of mtDNA-encoded genes, though NAD levels were not determined (Gomes et al., 2013). Though isoforms of NMNAT are responsible for the final step of amidated NAD synthesis in the nucleus, Golgi apparatus, and mitochondria, the catalytic efficiency of these purified enzymes is approximately forty times higher than that of the other key player in the NAD salvage pathway, Nampt (Berger et al., 2005; Revollo et al., 2004). Thus, overexpression of the latter rate-limiting enzyme is more likely to increase the conversion of NAM to NAD in most tissues.

The intrinsic mechanisms that regulate the turnover of metabolites in the NAD salvage pathway remain poorly understood. However, increases in the expression of Nampt in tissues such as the liver and muscle forms at least one likely mechanism, as multiple environmental stimuli, including CR, exercise stress, and circadian cues, trigger this response (Costford et al., 2010; Peek et al., 2013; Song et al., 2014). Though not a true nicotinamidase, Nampt catalyzes a reaction that may be considered most closely analogous to that of yeast Pnc1, since it serves to increase the ratio of NAD to NAM. The dramatic longevity benefit of yeast overexpressing Pnc1 hints that increased Nampt expression may offer similar stress resistance (Anderson et al., 2002). Given these observations and the apparent impact of NMN supplementation on the oxidative metabolism of multiple mammalian tissues, overexpression of the NMN-generating enzyme appears to be a viable strategy for recapitulating such metabolic advantages without the need for
dietary modification. This is especially true for scenarios in which the intracellular NAD level may be compromised, such those found during natural aging.

The recurring observation that steady state levels of NAD decline in muscle, liver, skin, and brain tissue of elderly mice and humans suggests that common mechanisms are in play (Braidy et al., 2011; Gomes et al., 2013; Massudi et al., 2012; Yoshino et al., 2011). However, it is unclear whether the effect is driven primarily by increased consumption or decreased synthesis of NAD. In the former case, the loss of NAD fits comfortably into the existing free radical theory of aging, if chronic PARP activation can be demonstrated to result from the age-related accumulation of oxidative damage to DNA. At least one group has claimed this observation in muscle (Mouchiroud et al., 2013). However, others have argued that NAD synthetic pathways may slow over time, noting a strong and unexplained trend toward decreased Nampt protein in the muscle and white adipose of old mice, which might effectively limit the rate of NAD recycling (Yoshino et al., 2011). In the latter scenario, the causal event is relatively easy to model in vivo by using genetic techniques to selectively delete the enzymes of the NAD salvage pathway or express hypomorphic alleles in young mice. However, several attempts to pursue these approaches have met with limited success, due to the embryonic or perinatal lethality resulting from whole body deletion of NMNAT1, NMNAT2, or Nampt (Conforti et al., 2011; Gilley et al., 2013; Revollo et al., 2007a). While deletion of the mitochondrial NMNAT3 is not lethal, this isoform appears to be essential only to the metabolism of erythrocytes, exerting no direct effect on liver or muscle NAD pools (Hikosaka et al., 2014). Nonetheless, the creation of floxed alleles for these genes creates myriad opportunities for investigating NAD restriction on a tissue-by-tissue basis, using lineage-specific Cre drivers to circumvent survival challenges. Such studies may serve to highlight unforeseen interdependencies between tissues and isolate the critical sites of action for NAD-boosting therapeutics.
**Goals of This Work**

The fact that nutrient restriction improves the probability of survival for both yeast and mammals is a testament to the many core elements of our metabolic machinery conserved from single-celled ancestors. However, rigorous experimentation is necessary to determine the relevance of specific biological pathways, such as those governing NAD synthesis, to more complex organisms. It is also important to consider that specific germline mutations and systemic NAD precursors, which appear to benefit muscle physiology in certain contexts, do not necessitate a muscle-autonomous mechanism of action.

The work presented herein aims to elucidate the ability of a single biosynthetic enzyme, Nampt, to impact the performance of skeletal muscle in youth and old age. By utilizing transgenic techniques to increase and eliminate the expression of Nampt protein in rodent muscle, these experiments isolate the influence of muscle-autonomous NAD synthesis on the contractile performance and oxidative capacity of the tissue under various conditions. The experiments test the hypotheses that 1) Nampt expression is sufficient to enhance the oxidative capacity of muscle in young mice, 2) muscle Nampt expression is sufficient to mitigate the age-related loss of exercise capacity, and 3) muscle NAD homeostasis is required to maintain muscle mass and contractile function. The associated findings provide insights into the impact of aging on muscle metabolism, with implications for the treatment of diverse myopathic states.
Figure 1.1. *The dual nature of NAD as a cofactor and substrate*. Left: NAD is continuously degraded to NAM as a substrate of four primary classes of NAD-dependent enzymes. The NAD salvage pathway recombines NAM with PRPP and ATP (not shown) to reform NAD through a two-step process mediated by Nampt (highlighted) and NMNAT, respectively. NR may enter the salvage pathway as NMN after being phosphorylated by dedicated kinases. Right: NAD is continuously reduced by glycolysis and the TCA cycle to provide NADH to complex I of the ETC, allowing the bulk of ATP synthesis to be achieved through OXPHOS. The oxidation and reduction of NAD affects only a single covalent bond, preserving the remaining structure of the dinucleotide. In most mammalian cells, the NAD salvage pathway must constantly regenerate NAD to support the redox-dependent reactions of ATP synthetic pathways.
CHAPTER 2: Increasing NAD Synthesis in Muscle via Nicotinamide

Phosphoribosyltransferase is not Sufficient to Promote Oxidative Metabolism
ABSTRACT

The NAD biosynthetic precursors nicotinamide mononucleotide and nicotinamide riboside are reported to confer resistance to metabolic defects induced by high fat feeding, in part by promoting oxidative metabolism in skeletal muscle. Similar effects are obtained by germline deletion of major NAD-consuming enzymes, suggesting that the bioavailability of NAD is limiting for maximal oxidative capacity. However, due to their systemic nature, the degree to which these interventions exert cell or tissue-autonomous effects is unclear. Here, we report a tissue-specific approach to increase NAD biosynthesis only in muscle by overexpressing nicotinamide phosphoribosyltransferase, the rate-limiting enzyme in the salvage pathway that converts nicotinamide to NAD (mNampt mice). These mice display a ~50% increase in skeletal muscle NAD levels, comparable to the effects of dietary NAD precursors, exercise regimens, or loss of poly-ADP ribose polymerases (PARPs), yet surprisingly, do not exhibit changes in muscle mitochondrial biogenesis or mitochondrial function and are equally susceptible to the metabolic consequences of high fat feeding. We further report that chronic elevation of muscle NAD in vivo does not perturb the NAD/NADH redox ratio. These studies reveal for the first time the metabolic effects of tissue-specific increases in NAD synthesis and suggest that critical sites of action for supplemental NAD precursors reside outside of the heart and skeletal muscle.

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is a ubiquitous pyridine nucleotide that functions as an essential reduction-oxidation (redox) cofactor in cellular metabolism. Recent years have seen growing interest in the expanded roles for NAD as a co-substrate in a myriad of signaling contexts, spurred in part by the discoveries that, in yeast, enhancing flux through the NAD salvage pathway is necessary and sufficient for lifespan extension by calorie restriction (CR) and that in higher eukaryotes, NAD levels fluctuate in response to circadian cues, exercise, and nutritional status (Canto et al., 2009; Cantó et al., 2010; Nakahata et al., 2009). In mammalian
cells, NAD is continuously consumed as a substrate in ADP-ribosylation, cyclization, and deacylation reactions that influence a host of physiological processes (Belenky et al., 2007b). These functions highlight the necessity for NAD biosynthetic pathways in the maintenance of cellular homeostasis and suggest that modulation of the NAD pool might have therapeutic applications.

While the de novo synthesis of NAD from dietary tryptophan, nicotinic acid, or nicotinamide-containing nucleotides can contribute to the intracellular NAD pool in mammals, the constitutive degradation of NAD to nicotinamide (NAM) by endogenous enzymes renders a functional NAD salvage pathway necessary for life (Revollo et al., 2007a). The rate-limiting enzyme in the NAD salvage pathway, nicotinamide phosphoribosyltransferase (Nampt/NamPRTase/PBEF/Visfatin), catalyzes the condensation of NAM and phosphoribosyl pyrophosphate (PRPP) to nicotinamide mononucleotide (NMN), which is subsequently converted to NAD by NMN adenyltransferases (NMNAT1-3)(Revollo et al., 2004).

NAD salvage is at least partly regulated at the level of Nampt protein expression, which increases in response to various stresses and disease states, including inflammation, hypoxia, and oncogenesis. Nampt overexpression is sufficient to elevate NAD concentration and confer cytoprotective effects upon cells in culture (Revollo et al., 2004; Rongvaux et al., 2008; Yang et al., 2007a) and in vivo (Hsu et al., 2009). In skeletal muscle, Nampt protein levels have been observed to more than double in response to chronic exercise or CR (Chen et al., 2008, 2008; Costford et al., 2010; Koltai et al., 2010; Song et al., 2014). Importantly, administration of the specific Nampt inhibitor FK866 to calorie-restricted rats can partially or fully revert several beneficial metabolic phenotypes to the ad libitum conditions (Song et al., 2014). Though Nampt maintains a critical co-substrate of the sirtuin family of deacylases and ADP-ribosyltransferases (Imai et al., 2000), the range of mechanisms through which it can influence mammalian physiology remain largely unresolved.
The observations that global increases in NAD availability appear beneficial to mammalian metabolic health, especially in the context of nutrient excess, do not reveal the particular cells or tissues responsible for the effects. Whole-body deletion of major NAD consumers, including CD38 (Barbosa et al., 2007), PARP-1 (Bai et al., 2011a), and PARP-2 (Bai et al., 2011b), provides protection against diet-induced obesity and metabolic disease, though it is difficult to determine whether these phenotypes derive from the increased availability of NAD versus the loss of signals that would otherwise arise from the deleted enzymes. Studies targeting NAD synthesis via the direct provision of the Nampt product, NMN, as well as its unphosphorylated relative, nicotinamide riboside (NR), have bolstered the case that higher intracellular NAD levels improve oxidative metabolism and ameliorate deleterious consequences of a high fat diet. Such NAD-boosting strategies have been associated with enhanced mitochondrial biogenesis and function in tissues where elevated NAD was observed, including liver (Yoshino et al., 2011), brown adipose (Cantó et al., 2012), and skeletal muscle (Barbosa et al., 2007). These findings coincide with enhancements in energy expenditure, thermogenesis, and endurance, as well as correction of respiratory and ultrastructural defects in mouse models of mitochondrial disorders (Cerutti et al., 2014a; Khan et al., 2014b). However, NAD-modulating interventions involving either germline genetic mutations or transient, systemically administered treatments cannot conclusively resolve the primary site of action for NAD, nor the contributions of individual tissues to the whole animal metabolic phenotype. Thus, a conditional transgenic approach is needed to specifically and chronically modulate NAD homeostasis in metabolically significant tissues.

In this study, we investigated the degree to which increasing NAD concentration exerts tissue-autonomous effects on oxidative metabolism in otherwise healthy muscle. By specifically overexpressing Nampt in muscle, we provide evidence that a modest increase in the steady-state level of NAD is insufficient to improve muscle oxidative function or ameliorate consequences of high fat diet feeding, and does not alter the NAD/NADH ratio. The lack of phenotype in our
transgenic model suggests that NAD-boosting treatments may benefit muscle via signals that originate from outside the tissue.

**METHODS AND MATERIALS**

*Generation of muscle-specific Nampt transgenic mice*

A Cre-inducible Nampt construct was generated by placing a floxed transcriptional STOP element between a CAGGS promoter and the Nampt cDNA. This construct was then targeted to the Col1A1 locus as previously described (Firestein et al., 2008). Nampt$^{+/w}$ mice were generated by crossing Nampt$^{fl/w}$ or Nampt$^{fl/fl}$ mice to muscle creatine kinase (MCK)-Cre transgenic mice on the C57BL6/J background obtained from Jackson Labs. Nampt$^{+/w}$ mice were subsequently generated by crossing Nampt$^{+/w}$ to Nampt$^{fl/w}$ or Nampt$^{fl/fl}$ mice. Littermates lacking either MCK-Cre or the floxed allele were used as controls. Mice were studied between the ages of 3 and 9 months, as indicated. The NAM supplementation experiment in Figure 2.1I was performed in 15 month old animals.

*Animal housing and diets*

All mice were housed according to approved IACUC protocols and sacrificed after overnight fast. Animals were kept in a specific pathogen free barrier facility with controlled temperature and humidity and a 12h/12h light/dark cycle with lights on at 7 am. Mice were fed standard chow diet (CD) containing 120 ppm niacin (Rodent Diet 5010, LabDiet) unless otherwise noted. Obesity was induced with high fat diet (HFD) (D12492, Research Diets) containing 60% calories from fat. For NAM and NR supplementation experiments, compounds were dissolved weekly in the drinking water at 3.67 mg/mL in light-protected bottles.

*Immunoblots*
Tissues were lysed with a motorized homogenizer in lysis buffer (25mM Tris-HCl pH 7.9, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 1% v/v NP-40, 10% v/v glycerol) supplemented with Complete protease inhibitor cocktail (Roche). SDS-PAGE was performed on 4-15% Tris-HCl gels (Biorad) using 20 µg of cleared lysate boiled for 5 minutes in 1x Laemmli buffer. Gels were transferred to PVDF membranes (Millipore) and blocked with 5% non-fat milk. Primary antibodies were diluted 1/1000 in TBS-T according to manufacturer instructions. PBEF (A300-372A) was from Bethyl Laboratories, Mitoprofile total OXPHOS antibody cocktail (ab110413) was from MitoSciences, and Beta Actin-HRP (ab49900) was from Abcam. Antibody binding was detected using chemiluminescent horseradish peroxidase substrate (Perkin Elmer) and quantified using ImageJ software.

RNA and DNA extraction

RNA was isolated from ~25 mg of muscle tissue using 1 mL Trizol (Qiagen) reagent according to manufacturer instructions. For total DNA isolation, ~25 mg of tissue was incubated in digestion buffer (100 mM Tris-HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% w/v SDS, 1mM β-mercaptoethanol) supplemented with 0.3 mg/mL proteinase K (Sigma) overnight at 55°C, followed by an additional 0.3 mg/mL of proteinase K for 1 hour at 55°C. Nucleic acids were precipitated by addition of 1.25M NaCl and ethanol, washed in 70% v/v ethanol, and resuspended in Tris-EDTA buffer supplemented with 80 µg/mL RNaseA (Roche).

Quantitative real-time PCR and Recombination Analysis

One µg of total RNA was reverse transcribed using High Capacity cDNA reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR for gene expression was performed on an Applied Biosystems 7900HT system using 15 ng of cDNA per reaction and a standard curve method relative to reference gene 36B4. One control sample was excluded as an outlier according to the Grubb’s test with α=0.05 due to low reference gene expression. For LoxP recombination analysis, 15 ng of total DNA was analyzed by a custom TaqMan Copy Number
Assay (Life technologies) normalized to reference gene *Trfc*, with custom primers flanking the 3’ LoxP site of the transgene. For determination of mtDNA/nDNA content, 15 ng of total DNA was analyzed with primers specific to mitochondrial gene *ND1* (F: 5'-TAA CCG GGC CCC CTT CGA CC-3, R: 5-TAA CGC GAA TGG GCC GGC TG-3) and nuclear gene *TBP* (F: 5-CCC CTT GTA CCC TTC ACC AAT-3, R: 5-GAA GCT GCG GTA CAA TTC CAG-3).

**Glucose tolerance test**

Mice were injected intraperitoneally with D-glucose solution at a dose of 1.5 g/kg body weight after overnight fast. Injected mice were allowed free locomotion and access to water. Tail vein blood glucose was measured with a handheld glucometer (Abbott) for the next 2 hours.

**Citrate synthase activity**

Citrate synthase activity was assayed as described previously, with modifications (Trounce et al., 1996). 200 µL of reaction mixture (125 mM Tris-HCl pH 8, 0.3 mM Acetyl CoA, 0.1% v/v Triton X-100, 0.1 mM DTNB) was incubated with 5 µg of skeletal muscle protein lysate or 10 µg of isolated skeletal muscle mitochondrial protein at 30°C for 10 minutes. The reaction was initiated by the addition of freshly prepared 0.5 mM oxaloacetate and the reduction of 5’, 5’-dithiobis 2-nitrobenzoic acid (DTNB) was continuously monitored by absorbance at 412nm for 3 minutes.

**NAD metabolite extraction from muscle and mitochondria**

NAD, NAM, and NMN were extracted from ~50 mg of muscle tissue in 0.6 M perchloric acid at 4°C using a TissueLyzer (Qiagen) set to 20 Hz for 6 minutes. Lysates were pelleted at 20,000xg for 10 minutes at 4°C and diluted 1/200 in ice cold 100 mM phosphate buffer, pH8. For determination of NMN, the supernatant was further neutralized with 1 M potassium carbonate and centrifuged to remove insoluble material. NADH was similarly extracted from ~50mg of muscle tissue in alkaline extraction buffer (25 mM NH₄Ac, 25 mM NaOH, 50% v/v acetonitrile) flushed
with nitrogen gas. For measurement by cycling assay, alkaline lysates were mixed 1/1 v/v with ethanol extraction buffer (250 mM KOH, 50% v/v EtOH) and heated at 55°C for 10 minutes. Lysate supernatants were diluted 1/50 in ice-cold 100 mM phosphate buffer, pH8. NAD was extracted from 100 µg of isolated mitochondria by vortexing vigorously in 100 µL of 0.6M perchloric.

**NAD and NADH measurement**

NAD and NADH were immediately measured after extraction by enzymatic cycling assay in a 96-well format as described previously, with modifications (Graeff and Lee, 2002). Briefly, 5 µl of NAD standards or diluted tissue extracts were combined with 95 µl of cycling mix (2% ethanol, 100 µg/ml alcohol dehydrogenase, 10 µg/ml diaphorase, 20 µM resazurin, 10 µM flavin mononucleotide, 10 mM nicotinamide and 0.1% BSA in 100 mM phosphate buffer, pH 8.0). The cycling reaction proceeded for 30 minutes at room temperature while resorufin accumulation was measured by fluorescence excitation at 544 nm and emission at 590 nm. NAD and NADH values were confirmed by HPLC analysis of the same extracts.

**NAD metabolite measurement by HPLC**

Separation of NAD, NAM, and NADH was performed on non-diluted extracts as described (Little et al., 2010), with modifications, using an Adsorbosphere XL ODS column (5 µm, 4.6 x 250 mm) preceded by a guard column at 50°C. Flow rate was set at 0.4 mL/min. The mobile phase was initially 100% of mobile phase A (0.1 M potassium phosphate buffer, pH 6.0, containing 3.75% methanol). Methanol was linearly increased with mobile phase B (0.1 M potassium phosphate buffer, pH 6.0, containing 30% methanol) to 50% over 15 minutes. The column was washed after each separation by increasing mobile phase B to 100% for 5 min. UV absorbance was monitored at 260 and 340 nm with Shimadzu SPD-M20A. Separation of NMN was carried out on an YMC-Pack ODS-A column (5 µm, 4.6 x 250 mm) at 30°C. Flow rate was set at 0.4 mL/min. The mobile phase was initially 100% of mobile phase A (0.1 M potassium
phosphate buffer, pH 6.0) for first 8 min. Methanol was linearly increased with mobile phase B (0.1 M potassium phosphate buffer, pH 6.0, containing 30% methanol) to 50% over 7 minutes. The column was washed after each separation by increasing mobile phase B to 100% for 3 min and UV absorbance was similarly monitored. Pertinent peak areas were integrated by the LabSolution software from Shimadzu, quantified using standard curves, and normalized to weights of frozen tissues.

Mitochondrial isolation from skeletal muscle

Mice were euthanized by cervical dislocation and their hindlimb muscles dissected and placed immediately in ice-cold muscle homogenization buffer (50mM Tris-HCl pH 7.4, 100mM KCl, 5mM MgCl$_2$, 1mM EDTA pH 8.0, 1.8mM ATP) at pH 7.2. Entire procedure was performed at 4°C. The fat and connective tissues were removed and minced muscles were incubated for 2 minutes in 2.5mL of homogenization buffer supplemented with 60 U/mL of protease from Bacillus licheniformis (Sigma). Muscle pieces were washed twice with 5 mL unsupplemented homogenization buffer and subjected to an ice-cold motorized homogenizer for 10 minutes at 150 RPM. A small aliquot of the homogenate was removed and stored at -80°C for further analysis. The remaining homogenate was centrifuged at 720 x g for 5 minutes at 4°C. The pellet was resuspended in homogenization buffer and centrifuged for an additional 5 minutes at 720 x g. The supernatants were combined and centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in homogenization buffer and further centrifuged for 10 minutes at 10,000 x g. The final mitochondrial pellet was resuspended in resuspension buffer (225mM sucrose, 44mM KH$_2$PO$_4$, 12.5mM MgOAc, and 6mM EDTA, pH 7.4) at 4 μl of buffer per milligram of muscle tissue.

Mitochondrial oxygen consumption

Oxygen consumption was measured using the Oroboros High-Resolution Respirometry System (Innsbruck, Austria). Two 2 mL chambers were loaded with 150 μg isolated skeletal
muscle mitochondrial protein in 37°C MiRO5 respiration medium (110 mM sucrose, 20 mM HEPES, 10 mM KH$_2$PO$_4$, 20 mM taurine, 60 mM K-lactobionate, 3 mM MgCl$_2$•6H$_2$O, 0.5mM EGTA, 1 g/L defatted BSA, pH 7.1). Ten mM pyruvate and 5 mM malate or 20 µM palmitoyl carnitine and 5 mM malate were added as oxidative substrates, followed by 4 µmol of ADP to stimulate maximal respiration. Glutamate and cytochrome C were added to replenish TCA cycle intermediates and confirm the integrity of mitochondrial membranes, respectively, following manufacturer instructions. To interrogate complex II (succinate dehydrogenase), 1 µM rotenone plus 10 mM succinate was added. The assay was ended by addition of 5 µM antimycin A for calculation of residual oxygen consumption.

**Histology and Immunohistochemistry**

Histological analysis was performed on 4-micron thick paraffin sections, deparaffinized in histoclear (National Diagnostics) followed by rehydration through a graded ethanol series. Selected sections were stained with Gill 3 hematoxylin (Thermo Scientific) and eosin (Sigma). For immunohistochemistry, antigens were retrieved following rehydration by microwave heating in EDTA buffer (pH 7.5) for 10 minutes followed by cooling for 20 minutes and blocking of endogenous peroxidase with hydrogen peroxide. Slides were incubated overnight at 4°C with the primary antibody against slow skeletal myosin heavy chain (Abcam, ab11083) diluted 1:3000. Following washing with PBS, slides were incubated with biotinylated secondary antibody (Vector Laboratories, BA-2001) at 37°C for 1 hour, washed three times with PBS and incubated with streptavidin-conjugated HRP (Vector Laboratories, PK6101) for 1 hour at 37°C. Staining was detected with diaminobenzidine (Dako) chromogen and slides were counterstained with hematoxylin.

**Treadmill, voluntary wheel running, and grip strength**

Mice were habituated to the Exer3-6 treadmill system with shock detector (Columbus Instruments; Columbus, OH) by running at 10 m/min for 15 minutes at 0° incline one day before
the test. To test exercise tolerance, mice were fasted with access to water for 3 hours mid-day before simultaneously running a protocol described in Figure 2.3D from 0-25 m/min at 0° incline. Exhaustion was defined as 50 cumulative electrical stimuli (73 V, 0.97 mA, 1 Hz). Exhausted mice were removed from the treadmill and allowed access to food and water. To assess voluntary running, mice were individually housed and allowed access to a computer-monitored running wheel (Columbus Instruments) continuously for 3 weeks. Data were compiled from the second and third week after habituation. Grip strength was measured with a rodent grip meter (TSE; Hamburg, Germany) as described previously (Akpan et al., 2009).

*Indirect calorimetry*

$VO_2$, $VCO_2$, and activity were measured in individually housed mice using an open circuit calorimeter (CLAMS; Columbus Instruments). Room air was supplied at 0.6 L/min and exhaust air was sampled at 1 hour intervals for 96 hours. RER was calculated as the ratio of $VCO_2/VO_2$, with lower values indicating oxidation of fatty acids. Activity was monitored by infrared beam interruption. Data were compiled from hours 48-96 after habituation.

*Serum NEFA and cholesterol*

Plasma samples were collected from the tail veins of awake mice after a 5 hour fast. Plasma lipids were analyzed individually by analytical chemistry with an automated Cobas Mira Autoanalyzer (Roche Diagnostic Systems).

*Lactate, pyruvate, and ketone measurements*

The concentrations of lactate, pyruvate, β-hydroxybutyric acid and acetoacetate were assayed by GC/MS as their acetylated-pentafluorobenzyl derivatives as described (Tomcik et al., 2011), with modifications. The derivatized analytes were quantified against an internal standard of $[d_5]$ beta-hydroxypentanoate ($d_5$-BHP). Frozen tissues (~40mg) were spiked with 20 nmol $d_5$-BHP and 20 µmol NaB$_3^2$H$_4$ and homogenized on ice for 2 minutes in 0.5 mL of a solvent mixture
containing equal parts acetonitrile, methanol, and water, as described (Des Rosiers et al., 1988). The cold homogenate was then centrifuged at 3000 xg for 10 minutes at 4°C. The supernatants were reacted with 20 µmol pentafluorobenzyl bromide (PFBBBr) in acetone solution at 60°C for 1 hour. The PFBBBr derivatized analytes were extracted with 1 mL chloroform, dried with nitrogen gas, and further derivatized by acetylation using 30µL acetic anhydride and 30µL of pyridine for 1 hour at 65°C. GC/MS analysis was carried out on an Agilent 5973 mass spectrometer linked to a Model 6890 gas chromatograph equipped with an autosampler and an Agilent OV-225 capillary column (30 m, 0.32 mm inner diameter). The injection volume was 1 uL with a 30/1 split ratio and the carrier gas was helium at a 2 mL/min. The injector temperature was set at 200°C and the transfer line at 250°C. The GC temperature program was as follows: 100°C for 1 minute, increase by 10°C /min to 230°C, followed by 50°C /min to 300°C, and hold for 5 minutes. Chemical ionization GC/MS was monitored for lactate, pyruvate, β-hydroxybutyric acid and acetoacetate at m/z ratios of 131, 132, 145, and 146, respectively.

**Estimation of compartmental free NAD/NADH ratios**

Estimates of NAD/NADH ratios in cytosol and mitochondria were calculated from metabolites presented in Table 2.1 (extracted from whole-tissue) according to the equilibrium constants for NAD-linked lactate dehydrogenase and β-hydroxybutyrate dehydrogenase, respectively, as determined previously (Williamson et al., 1967). Calculations according to the following equations assumed enzymatic equilibrium at 38°C and pH 7.0 in each compartment:

\[
\frac{[NAD^+]_{cyto}}{[NADH]_{cyto}} = \frac{[pyruvate][H^+]}{[L - lactate][1.11 \times 10^{-11}]}
\]

\[
\frac{[NAD^+]_{mito}}{[NADH]_{mito}} = \frac{[acetoacetate][H^+]}{[D - \beta - hydroxybutyrate][4.93 \times 10^{-9}]}
\]
Statistics

Results were analyzed by two-tailed unpaired Student's t-test or one-way ANOVA with Bonferroni's Multiple Comparisons post-hoc test, as indicated. \( P<0.05 \) was taken to be significant. Analysis was conducted with Graphpad Prism 5 software.

RESULTS

Generation of mNampt mice

To investigate the role of NAD in muscle, we generated mice carrying an exogenous copy of the Nampt cDNA separated from the CAGGS promoter by a floxed STOP cassette. Expression of Cre recombinase under control of the muscle creatine kinase promoter (MCK-Cre) results in excision of the STOP cassette and overexpression of Nampt in skeletal and cardiac muscle (mNampt mice). An important advantage of targeting muscle is that it avoids complications associated with the reported production of extracellular Nampt (eNampt/PBEF/Visfat) by adipose (Romacho et al., 2013), immune cells (Friebe et al., 2011), and hepatocytes (Garten et al., 2009). Deletion of the STOP cassette was detected in approximately 40% of muscle nuclei using a probe specific to the intact allele (Figure 2.1A), consistent with efficient recombination of the genomes contained within myofibers (Bothe et al., 2000). Expression of Nampt mRNA increased approximately 10- and 20-fold in the quadriceps of animals carrying one (NC) or two (NNC) copies of the transgene, respectively, while expression of the 3' UTR of endogenous Nampt was unaffected (Figure 2.1B). Similar increases in Nampt expression were confirmed in gastrocnemius and the more oxidative soleus muscles (data not shown). Transcripts of enzymes downstream in the NAD salvage pathway were unchanged, although expression of both NMNAT1 and NMNAT3 trended higher with increasing Nampt gene dosage (Figure 2.1B). Expression of the transcript for nicotinamide N-methyl transferase (NNMT), which competes with Nampt to metabolize NAM, was low in skeletal muscle and unaffected by
genotype. In accordance with Nampt transcript levels, we observed a robust increase in Nampt protein in both skeletal (~10-fold) and cardiac (~4-fold) muscles of NC mice (n=11 and 3, respectively) and an approximately 50% increase in intramuscular NAD content (Figure 2.1C-E). Because only a small number of NNC mice were available, we focused primarily on the NC mice for subsequent experiments.

Histological analysis of hindlimb muscles containing primarily glycolytic type IIB (extensor digitorum longus) and oxidative type I and IIA fibers (soleus) showed normal polygonal cross-sectional morphology and diameter, as well as equivalent staining for type I myosin heavy chain (MyHCI) in NC mice as compared to littermate controls (Figure 2.1F). NAD levels in heart were also significantly elevated, albeit to a lesser degree than in skeletal muscle, consistent with the activity of MCK-Cre in these tissues (Brüning et al., 1998) and the relative expression of Nampt (Figure 2.1G). Gross examination of myocardium did not reveal any major changes in heart tissue, nor was there a change in organ weight (Figure 2.1H). To test whether NAD generation in mNampt mice is limited by NAM availability, we administered 400 mg/kg NAM in the drinking water for one week and examined skeletal muscle NAD levels. No further elevation of NAD was observed in mNampt mice under these conditions, suggesting that NAM availability does not limit Nampt activity in muscle under standard dietary conditions (Figure 2.1I).

Characterization of muscle oxidative function

Based on earlier work employing NAD-boosting strategies in mice, we hypothesized that skeletal muscle of mNampt mice would exhibit enhanced oxidative function. To test this, we examined several markers of mitochondrial content in gastrocnemius and quadriceps muscles. We found protein expression of subunits from four different complexes of the electron transport chain to be similar in control and mNampt mice (Figure 2.2A). We further observed similar levels of citrate synthase activity (Figure 2.2B) and mitochondrial DNA (mtDNA) content (Figure 2.2C) between the two groups. Consistent with these findings, the Nampt transgene had no significant effect on the expression of genes associated with mitochondrial biogenesis (TFAM, PGC1a,
mfsn2, Sirt1), oxidative phosphorylation (CytC, Cox5b, Ndufs8), coupling (UCP2), or substrate selection (PDK4, MCAD, Sirt3) in quadriceps or soleus muscles (Figure 2.2E).

Interventions that alter NAD in cultured cells or the liver have been reported to concomitantly alter the mitochondrial NAD pool (Cantó et al., 2012; Yang et al., 2007a), which may directly influence oxidative metabolism. We thus hypothesized that mitochondria isolated from the muscles of mNampt mice would exhibit an increase in NAD content proportional to the effect in whole muscle tissue. Surprisingly, we found that the NAD content of muscle mitochondria was similar between groups (Figure 2.2D). Accordingly, mitochondria did not exhibit any alterations in oxidative capacity that persisted through isolation, as detected using high-resolution respirometry (Figure 2.2F and G). Mitochondria isolated from control and mNampt muscle showed similar patterns of oxygen consumption across all complexes on both pyruvate and fatty acid substrates. These results suggest that Nampt overexpression is not sufficient to promote the biogenesis or respiratory capacity of muscle mitochondria.

We next sought to test the influence of Nampt on muscle performance in vivo. We found voluntary ambulatory and wheel running activity across the light/dark cycle to be indistinguishable between control and NC mice (Figure 2.3A and B). Forelimb strength and treadmill running capacity were also comparable (Figure 2.3C-E). Together, these data indicate that NAD is unlikely to limit muscle metabolic function in healthy wild type mice.

Muscle Nampt does not protect against metabolic consequences of high fat diet- Genetic and pharmacological interventions that elevate NAD levels have been reported to ameliorate many of the negative consequences of high fat diet feeding, including insulin resistance and weight gain. To investigate whether Nampt-mediated NAD synthesis in muscle conferred similar protective effects, we placed mNampt mice on a high fat diet (HFD) for 24 weeks. No significant differences in weight gain, plasma free fatty acids, or total cholesterol were observed between control and NC mice (Figure 2.4A-C). After 10 weeks on respective diets, HFD-fed NC and control mice exhibited similar declines in glucose tolerance, VO2, and RER, as compared to
chow-fed littermates (Figure 2.4D and E, 2.4G-J). Interestingly, HFD caused a slight but significant decline in intramuscular NAD in control mice that was completely rescued by the transgene (Figure 2.4F). Thus, intramuscular NAD levels do not appear to have a major influence on the general metabolic deterioration caused by HFD.

Nampt overexpression in muscle does not significantly affect steady-state levels of NAM or NMN- NAM, the substrate of Nampt, is a non-competitive inhibitor of sirtuins and a competitive inhibitor of PARPs. Thus, one mechanism by which Nampt overexpression might influence such enzymes is through the depletion of NAM, rather than through the generation of NAD per se. Contrary to our initial expectation, we did not observe a significant depletion of NAM in quadriceps muscles of mNampt mice (Figure 2.5A), potentially indicating that this metabolite is rapidly replenished. We also found no significant accumulation of NMN, the direct product of Nampt, in mNampt muscle (Figure 2.5B), supporting the view that Nampt catalyzes the rate-limiting step in the NAD salvage pathway.

Muscle redox state is independent of Nampt-mediated NAD synthesis- The reduced form of NAD, NADH, acts as a key electron donor and allosteric regulator of mitochondrial dehydrogenases, and the ratio of oxidized to reduced nucleotides can influence the rates of many reactions within the cell. While overexpression of Nampt in cells and treatment of mice with NR have both been reported to increase the NAD/NADH redox ratio (Cantó et al., 2012; Cerutti et al., 2014a; Fulco et al., 2008), it remains unresolved whether changes in absolute NAD concentration or the redox ratio play a greater role in mediating the effects of NAD-boosting interventions. Interestingly, total concentrations of NADH were significantly elevated in the muscles of mNampt mice, reflecting an apparent equilibration between the oxidized and reduced forms of NAD to maintain the NAD/NADH ratio across a range of absolute concentrations (Figure 2.5C and D).

A large fraction of the intracellular NADH pool is protein-bound and therefore inaccessible to enzymatic reactions that would lead to its oxidation. NAD(H) is further compartmentalized into mitochondrial and nucleo-cytosolic pools, suggesting that significant changes in the abundance of
free nucleotides within a given compartment could be missed by measuring only total tissue levels. To confirm the maintenance of redox state in the nucleo-cytosolic compartment of mNampt muscle, we determined the concentrations of lactate and pyruvate, which can be used to estimate the ratio of free NAD/NADH in the cytosol via the equilibrium constant for lactate dehydrogenase (Table 2.1, Figure 2.5G). To test whether redox was altered in the mitochondrial compartment, we determined the concentrations of the ketone bodies β-hydroxybutyrate and acetoacetate and estimated the free NAD/NADH ratio via the equilibrium constant for β-hydroxybutyrate dehydrogenase (Table 2.1, Figure 2.5H). Consistent with measurements of total metabolite pools, Nampt overexpression was not found to significantly affect the redox state of either compartment.

DISCUSSION

Chronic deficiency of dietary NAD precursors is known to cause the progressive and lethal disease Pellagra (Bogan and Brenner, 2008), but the more recent notion that increasing NAD availability might suffice to enhance the metabolic fitness of an otherwise healthy tissue is just beginning to be explored. We report that targeting the NAD salvage pathway in muscle via overexpression of Nampt increases the NAD content of skeletal muscles by approximately 50%, but does not overtly influence muscle physiology or whole body energy balance in the context of standard or high fat diets. Our findings represent the first investigation of these phenotypes using a tissue-specific approach to enhance NAD synthesis and stand in stark contrast to the beneficial effects that have been observed using a variety of genetic and pharmacological approaches to elevate NAD throughout the whole body.

It is noteworthy that our model achieved a relative elevation of skeletal muscle NAD greater than or equal to those reported for small molecule precursor supplementation (Cantó et al., 2012; Gomes et al., 2013), exercise (Canto et al., 2009; Cantó et al., 2010), or deletion of
either PARP-1 or PARP-2 (Bai et al., 2011a, 2011b). Since increased NAD was achieved via supraphysiological levels of Nampt protein expression and was not further enhanced by supplementing NAM, it is possible that negative feedback maintains the steady-state NAD concentration in our model at or near a theoretical limit for salvage-mediated synthesis. These observations are consistent with reports that the $K_m$ of Nampt for NAM in the presence of ATP is well below our observed tissue concentrations of NAM and that physiological levels of pyridine nucleotides can inhibit Nampt activity in rat liver extracts (Burgos and Schramm, 2008; Dietrich et al., 1968). Despite the elevated steady-state concentration of muscle NAD that was achieved, mNampt mice failed to display phenotypes suggesting any alteration of mitochondrial content (Figure 2.2A-C) or transcriptional adaptations (Figure 2.2E), consistent with their unchanged strength and exercise capacity (Figure 2.3B-E). In addition, mNampt mice gained weight normally on a high fat diet and did not display changes in glucose tolerance or oxygen consumption (Figure 2.4E and H). While obese control animals exhibited diminished metabolic health coincident with a small but significant decline in skeletal muscle NAD, this decline was completely rescued by the Nampt transgene to no effect (Figure 2.4F). Thus, the slight decrease in muscle NAD does not contribute meaningfully to the detrimental consequences of obesity, nor does elevating NAD have a protective effect.

While little is known about the dynamics of pyridine nucleotide transport across the mitochondrial membranes, it is appreciated that mitochondria maintain bioenergetics via a distinct NAD pool that is reduced to NADH in a much higher proportion than the nucleo-cytosolic pool and is shielded from the activity of nuclear and cytosolic enzymes (Di Lisa and Ziegler, 2001; Pittelli et al., 2010; Yang et al., 2007a). Though distinct, the mitochondrial NAD pool tends to mirror expansion or contraction of the total NAD pool in cultured cells and in the liver, where NAD deficiency can limit mitochondrial oxidative metabolism (Cantó et al., 2012; Peek et al., 2013). While we observed a slight trend toward an increase in mitochondrial NAD content in muscle (Figure 2.2D), the effect was clearly dampened compared to the increase in whole tissue. Moreover, mitochondria isolated from mNampt muscle consumed pyruvate and palmitoyl
carnitine at equivalent rates to those of control littermates (Figure 2.2G), consistent with the VO$_2$ and RER measurements from whole animals (Figure 2.4H and J). Since supplemented NAD precursors can theoretically bypass any compartmentalization of Nampt activity, it is plausible that their beneficial effects reflect a more pronounced influence on the mitochondrial pool. However, we found that administration of NR at 400 mg/kg, either as a single intraperitoneal bolus or a daily dose in the drinking water for one week, significantly increased neither mitochondrial nor whole tissue NAD levels (unpublished results). While an increase in whole muscle NAD was previously reported following NR supplementation in the chow (Cantó et al., 2012), the discrepancy is unlikely to be caused by the route of administration, since a more recent study employing NR-supplemented chow for up to 16 weeks also found non-significant changes in muscle NAD (Khan et al., 2014b). Thus, the available evidence suggests that NR exerts only a mild influence on skeletal muscle NAD content in young, healthy animals, and it remains to be determined whether specific expansion of the mitochondrial or cytosolic NAD pools directly influences oxidative metabolism in this tissue.

We found intramuscular NMN content to be ~50-100-fold lower than that of NAD across genotypes and relatively unaffected by transgene expression, consistent with the rate-limiting nature of Nampt in the NAD salvage pathway (Figure 2.5B). However, Nampt also failed to have a prominent effect on the intramuscular concentrations of its substrate, NAM (Figure 2.5A). This is of interest because it has been proposed that NAM acts as a feedback inhibitor of NAD-dependent enzymes in the concentration range that we have detected (Anderson et al., 2003; Bitterman et al., 2002; Schmidt et al., 2004). Indeed, evidence indicates that depletion of NAM by the Nampt analogue Pnc1 is a key mechanism regulating sirtuins in yeast (Anderson et al., 2003). In mouse muscle, however, our findings suggest that homeostatic mechanisms maintain approximately normal tissue NAM levels, as was observed in the context of PARP-1 deletion (Bai et al., 2011a). Our results may indicate that a) flux through the salvage pathway is unchanged, b) increased NAM consumption is counterbalanced by a corresponding increase in NAM generation.
by NAD-dependent enzymes, or c) NAM exchanges continuously with the plasma to maintain a stable tissue concentration, irrespective of Nampt activity.

The cellular NAD/NADH ratio is sensitive to metabolic flux and extrinsic factors, and can modify enzymatic activity independently from changes in NAD concentration per se (Fulco et al., 2008; Lin et al., 2004). For example, it has been suggested that the transcriptional co-repressor CtBP is reciprocally regulated by the oxidized and reduced nucleotides, and it is debated whether the sirtuins are competitively inhibited by NADH in vivo (Chen et al., 2008; Lin et al., 2004; Schmidt et al., 2004). Thus, any changes in the NAD/NADH ratio could mediate effects of NAD-boosting interventions. Contrary to our expectation that Nampt overexpression would increase the skeletal muscle NAD/NADH ratio, as reported in isolated myoblasts (Fulco et al., 2008) and heart (Hsu et al., 2009), as well as muscles of NR-fed mice (Cantó et al., 2012), we observed equilibration between the oxidized and reduced nucleotides to maintain the redox state in mNampt muscle (Figure 2.5D). Several key differences distinguish our experiment from previous studies. Firstly, experiments in cultured myoblasts may not accurately model the changes in nutrient availability, glycolytic flux, or ATP demand experienced by muscle fibers in vivo, all of which affect the redox state of NAD. Moreover, the redox states of liver and other tissues may influence muscle in vivo via circulating redox-active metabolites, including lactate, pyruvate, and ketone bodies. Secondly, in the cardiac model of Hsu and colleagues, Nampt overexpression shifted the redox ratio in concert with a larger increase in NAD than has been reported for any other intervention (~5-fold) (Hsu et al., 2009), yet a partial equilibration was still apparent. Lastly, the dietary delivery of NR is distinct from our genetic model in that it amounts to a series of discrete systemic administrations, which may be responsible for its effect on muscle redox state. Our finding that redox state was not chronically affected in mNampt mice indicates that the metabolic processes responsible for establishing the NAD/NADH ratio are rapid relative to the basal rate of NAD synthesis. Analogous observations were made following deletion of the circadian regulator Rev-erbα, in which the muscle NAD/NADH ratio was maintained in the context
of a diminished NAD pool (Woldt et al., 2013). Our data indicating that lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were unchanged in the muscles of transgenic mice (Figure 2.5G,H) provide additional evidence that NAD equilibrates with NADH and, insofar as the associated enzymes can be presumed to be in equilibrium, adds compartment-specific resolution to both nucleo-cytosolic and mitochondrial pools of the free nucleotides. Thus, homeostatic mechanisms appear to maintain the NAD/NADH ratio in muscle despite a chronic 50% increase in the size of the NAD pool.

It is interesting to consider why increased NAD availability did not have clear effects on skeletal muscle physiology. Unlike yeast, where sirtuins are the most prominent consumers of NAD (de Figueiredo et al., 2011), mammalian genomes encode several additional classes of NAD-consuming enzymes that play important roles in signaling and metabolism and depend on the same NAD pool (Berger and Ziegler, 2004). The evolution of different classes of NAD-dependent enzymes was likely accompanied by additional layers of regulation responsible for modifying the enzyme activities in different contexts. In support of this view, the best-studied mammalian sirtuin, SIRT1, is now appreciated to have numerous binding partners and post-translational modifications (Revollo and Li, 2013), at least some of which can regulate deacetylase activity independently from changes in NAD (Gerhart-Hines et al., 2011). Therefore, it will be important to identify the specific tissues or cell types that are capable of responding directly to changes in NAD availability.

Overall, our findings using a conditional Nampt allele support a model in which the effects of globally increasing NAD availability on muscle physiology are not muscle-autonomous. It remains possible that more profound muscle-autonomous effects would emerge following larger increases in NAD, concurrent energetic stress, or in a diseased state. However, we note that none of these conditions were required for NR supplementation or PARP-1 deletion to benefit muscle. Interestingly, overexpression of the NAD-dependent deacetylase SIRT1 in the brain has recently been shown to improve muscle physiology (Ramadori et al., 2011; Satoh et al., 2013),
whereas overexpression of the enzyme in the muscle itself was ineffective (White et al., 2013). Moreover, NMN can promote insulin secretion in cultured islets and in vivo, consistent with the stimulation of SIRT1 in pancreatic β cells (Revollo et al., 2007a; Yoshino et al., 2011), while knockdown of NNMT was recently shown to elevate adipose tissue NAD levels in concert with increased whole-body energy expenditure (Kraus et al., 2014). Thus, it is possible that improvements in muscle physiology during NAD precursor supplementation occur via pleiotropic effects of NAD-dependent enzymes in tissues other than skeletal muscle. It will be important to determine in future studies whether the central nervous system, hormones, circulating metabolites, or a combination thereof mediates the effects of global NAD availability on muscle physiology. It will also be interesting to determine the tissue-autonomous impact of enhanced NAD salvage capacity under conditions known to deplete the metabolite, including genotoxic stress (Yang et al., 2007a) and the course of natural aging (Gomes et al., 2013; Yoshino et al., 2011).

In summary, our muscle-specific transgenic approach provides an important step toward dissecting the mechanisms by which NAD availability influences mammalian physiology. A deeper understanding of the tissues and signals that mediate these effects will greatly facilitate the development of NAD-boosting therapeutics to combat comorbidities of obesity and aging.
Table 2.1. Concentrations of redox-sensitive metabolites in quadriceps muscle of 5-month-old mice. Concentrations presented as mean nmol/mg tissue (SEM). β-HB, β-hydroxybutyrate. No significant differences were found between genotypes by one-way ANOVA.
Figure 2.1. Overexpression of Nampt increases NAD concentration in muscle.
A, Proportion of Cre-recombined genomes in total genomic DNA of quadriceps muscle from mice carrying MCK-Cre in addition to one (NC) or two (NNC) copies of the Nampt transgene, as compared to littermates carrying the same number of copies, but lacking MCK-Cre (n=4-9 per group).

B, mRNA expression in quadriceps of NAD salvage pathway enzymes measured by qRT-PCR: total nicotinamide phosphoribosyltransferase (Nampt), 3’ untranslated region of endogenous Nampt transcript (Nampt3UTR), nicotinamide mononucleotide adenylyltransferases (NMNAT1-3), and nicotinamide N-methyltransferase (NNMT) (n=3-6 per group).

C, Western blot and D, quantification of Nampt protein in gastrocnemius muscle and heart of each genotype (n=3 per group).

E, NAD content of quadriceps muscle (n=4-11 per group).

F, Representative histological sections of extensor digitorum longus (EDL) and soleus muscles stained with H+E or antibody against myosin heavy chain I (myosin I). Scale bars represent 50 microns.

G, NAD content in whole hearts and H, heart weight normalized to tibia length (n=8-9 per group).

I, NAD content of quadriceps muscle from 15 month old mice supplied with 400 mg/kg/day NAM in the drinking water for two weeks. Data are expressed as the mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 by Student’s t-test (D and G) or one-way ANOVA with Bonferroni’s Multiple Comparisons Test (B and E). Mice were aged 5-10 months and fed a standard diet unless otherwise noted.
Figure 2.2. Nampt overexpression does not improve muscle oxidative function.

A, Western blot for representative components of the electron transport chain complexes in gastrocnemius muscle.

B, Citrate synthase activity in whole tissue lysates of quadriceps muscle. (n=3-4 mice per group, A.U. – arbitrary units).

C, Relative mtDNA/nDNA ratio in quadriceps muscle (n=7 per group).

D, NAD content in mitochondria isolated from pooled hindlimb muscles (n=4 per group).

E, mRNA expression of transcripts related to oxidative function in soleus and quadriceps (quad) muscle measured by qRT-PCR (n=6 per group).

F, Representative trace of oxygen consumption rate (OCR, left axis) and chamber oxygen concentration (right axis) of mitochondria isolated from control skeletal muscle. Arrows indicate sequential addition of malate (Mal), pyruvate (Pyr), adenosine diphosphate (ADP), glutamate
(Glu), cytochrome C (CyC), rotenone (Rot), succinate (Suc), and antimycin A (AnA), as well as opening and closing of the chamber to atmospheric oxygen.

G, Oxygen consumption rate (OCR) of mitochondria isolated from skeletal muscle and provided with respective substrates and inhibitors (n=4 per group). White and grey bars represent control and NC groups, respectively. Data are expressed as the mean +/- SEM. No changes are significant by Student's t-test. Mice were aged 5 to 7 months.
Figure 2.3. *Overexpression of Nampt in muscle does not change voluntary movement or endurance.*

A, Voluntary ambulatory activity assessed by counting infrared beam breaks. Timecourse represents the 48 hour period following habituation. Shaded panels indicate periods of darkness. Inset: average activity during light and dark hours of timecourse (n=5 mice per group).

B, Voluntary wheel running activity. Timecourse represents the first 96 hour period following habituation. Inset: average wheel revolutions during light and dark hours over the entire 14 day period (n=8 per group).

C, Maximum forelimb grip strength (n=9-12 per group).

D, Treadmill protocol for exercise tolerance test at 0° incline. Dashed line indicates presence of electrical stimuli.
Exercise-induced fatigability. Dashed line indicates the exhaustion threshold. Inset: average total distance run at exhaustion (n=10-11 per group). Blue and red bars represent control and NC groups, respectively. Data are expressed as the mean +/- SEM. No significant differences were observed at any time point by Student’s t-test. Mice were aged 9 months (A) or 4 to 5 months (B-E).
Figure 2.4. Overexpression of Nampt in muscle does not protect against metabolic consequences of high fat diet.
A, Body weights of mice provided a standard chow diet (CD) or high fat diet (HFD) for 24 weeks starting at 3 months of age (n=6 mice per group).

B, C, Plasma non-esterified fatty acids (NEFA) and total cholesterol after 24 weeks on the indicated diets (n=7-10 per group).

D,E, Glucose tolerance after 20 weeks on the indicated diets (n=6 per group).

F, NAD content of gastrocnemius muscles after 24 weeks on the indicated diets (n=5-6 per group).

G, Oxygen consumption (VO₂) normalized to body weight after 24 weeks on the indicated diets. Timecourse represents the 48 hour period following habituation. Shaded panels indicate periods of darkness (n=5 per group).

H, Average VO₂ during light and dark periods shown in G.

I, Respiratory exchange ratios over the same timecourse shown in G.

J, Average RER during light and dark periods shown in I. Data are expressed as the mean +/- SEM for each time point. *p<0.05, **p<0.01 by Student’s t-test.
Figure 2.5. Chronic elevation of NAD in muscle does not alter the NAD/NADH ratio.
A, Nicotinamide (NAM), B, nicotinamide mononucleotide (NMN), and C, NADH content of quadriceps muscle in 5 month old mice (n=4-11 mice per group).

D, Measured ratio of total NAD/NADH in quadriceps muscle. NAD values derived from figure 1D.

E, Measured ratio of lactate/pyruvate and F, β-hydroxybutyrate/acetoacetate in quadriceps (n=3-7 per group).

G, Calculated free NAD/NADH ratio in the nucleo-cytosolic and H, mitochondrial compartments. Data are expressed as the mean +/- SEM. *p<0.05, ***p<0.001 by one-way ANOVA with Bonferroni’s Multiple Comparisons Test.
CHAPTER 3: Loss of NAD Homeostasis Leads to Progressive and Reversible Degeneration of Skeletal Muscle
ABSTRACT

The oxidation and reduction of NAD is fundamental to the catabolism of all metabolic fuels, yet the availability of intracellular NAD becomes limited during genotoxic stress and the course of natural aging. The point at which declining NAD restricts associated redox-dependent processes to negatively impact muscle physiology remains unknown. We examined this question by specifically disrupting the NAD salvage pathway in murine skeletal muscle by deletion of Nampt. Young knockout mice were found to tolerate a dramatic 85% decline in intramuscular NAD content without loss of spontaneous activity or treadmill endurance. However, aging these mice beyond early adulthood revealed the progressive development of muscle weakness and fiber atrophy that could be completely reversed by administration of the NAD precursor nicotinamide riboside. Additionally, lifelong overexpression of Nampt preserved muscle NAD levels and exercise capacity in aged mice, revealing the critical role of tissue-autonomous NAD homeostasis in maintaining muscle mass and function.

INTRODUCTION

The flow of carbon and energy through glycolysis and oxidative phosphorylation is dependent on the electron-shuttling nature of nicotinamide adenine dinucleotide (NAD). Thus, the balance between synthesis and degradation of intracellular NAD must be tightly controlled. Though much of the seminal work establishing NAD as a co-factor for reduction-oxidation (redox) reactions was performed decades ago, recent attention has shifted toward its role as a co-substrate for enzymes that break down the dinucleotide to nicotinamide (NAM) to create signaling metabolites or post-translationally modify protein substrates. The resulting NAD-dependent signaling networks modify chromatin and transcription factor dynamics, as well as the kinetics of numerous enzymes to coordinate physiological responses to circadian rhythms and feeding status (Asher and Sassone-Corsi, 2015). Accordingly, localized restrictions in NAD bioavailability could potentially have profound effects on cellular function by dampening these signals or
crippling the production of ATP. Such restrictions have been reported during states of genotoxic stress that accompany a growing list of diseases, including cancer and neurodegeneration, as well as during the course of natural aging (Cantó et al., 2015).

Since NAD contains a NAM moiety that cannot be synthesized by most tissues de novo, the vast majority of mammalian cells must instead rely on a salvage pathway to locally regenerate degraded NAD or utilize NAM-containing precursors from the circulation. Intriguingly, an essential enzyme in this pathway, nicotinamide phosphoribosyltransferase (Nampt), as well as its product, nicotinamide mononucleotide (NMN), are found in both intracellular and extracellular compartments, suggesting a systemic element to the salvage and distribution of NAD (Revollo et al., 2007b). With this in mind, recent strategies to globally augment the NAD salvage pathway in rodents have employed the supplementation of NAM-containing compounds, including NMN and its unphosphorylated relative, nicotinamide riboside (NR), in the diet. The multitude of physiological benefits stimulated by these vitamins, including enhanced oxidative metabolism, synaptic plasticity, and insulin sensitivity, has been attributed to increased NAD levels in tissues such as the liver, brain, and skeletal muscle (Cantó et al., 2012; Gong et al., 2013; Yoshino et al., 2011). However, the specific sites to which intact NAD precursors are distributed and utilized in vivo have not been demonstrated experimentally.

Inherent to the concept of boosting NAD to gain a metabolic advantage is the notion that certain cells do not synthesize or access sufficient NAD to maintain optimal metabolic flux. This is proposed to be the case in mammals of advanced age, which have been reported by multiple groups to exhibit decreases in the NAD content of brain, liver, and muscle, coincident with declines in the function of these tissues (Braidy et al., 2011; Gomes et al., 2013; Mouchiroud et al., 2013; Stein and Imai, 2014b). We have previously shown that NAD levels do not limit the cardiac and skeletal muscle physiology of young, healthy mice (Frederick et al., 2015). However, we could not discount that the transgene might exert protective effects in the context of a specific challenge to NAD homeostasis, as potentially encountered during states of pathology or
advanced age. Furthermore, the lower threshold of NAD synthesis required to support NAD-dependent transcription and energetics in skeletal muscle has never been directly tested.

Here, we examine the consequences of restricting NAD availability in skeletal muscle by specific deletion of Nampt. We also examine the specific influence of the NAD precursor NR on muscle metabolism in states of compromised intramuscular NAD content. Our findings establish a previously unappreciated lower threshold of tolerability for NAD loss in muscle and shed light on potential mechanisms driving the age-related declines in muscle function and metabolic capacity.

METHODS AND MATERIALS

Generation of transgenic animals

Mice deficient for Nampt in skeletal muscle were generated by crossing Nampt^{fl/fl} mice (Rongvaux et al., 2008) to mice carrying Cre under control of the myosin light chain 1f (Mlc1f) promoter (Bothe et al., 2000). Mice homozygous for the Nampt transgene under expression of ckmm-Cre were generated as described previously (Frederick, 2015). All mice were on a C57BL6 background. Mice were housed in a temperature-controlled pathogen-free barrier facility on a 12 hour/12 hour light/dark cycle and fed a standard chow diet (Rodent Diet 5010, LabDiet) according to approved Institutional Animal Care and Use Committee protocols.

NR administration

For NR supplementation experiments, NR was dissolved weekly in the drinking water at 3.5 mg/mL and provided ad libitum in light-protected bottles. For NR^{m+2} experiments, custom synthesized isotopologue was dissolved in drinking water and administered to fed mice at a dose of 200 mg/kg by oral gavage. Animals were sacrificed after 100 minutes and tissues were harvested and snap frozen for metabolomics analysis.

NAD and ATP measurement
NAD was extracted from ~50 mg of snap frozen muscle tissue and 100 µg of purified muscle mitochondria in 0.5 mL and 0.1 mL of ice-cold 0.6 M perchloric acid, respectively. NAD content of extracts was measured using an enzymatic cycling assay and, for tissue samples, verified by HPLC, as described (Frederick et al., 2015). ATP was measured in neutralized acid extracts using the ATP Determination Kit (Life Technologies) according to manufacturer instructions.

Calorimetry and body composition

24-hour respiration, locomotion, and feeding activity of individually housed mice was assessed in an open-circuit indirect calorimeter (CLAMS, Columbus Instruments, OH) supplying room air at 0.6 L/min. RER was calculated from the ratio VCO₂/VO₂ and activity was determined by infrared beam breaks. Body composition was determined by nuclear magnetic resonance using a 3-in-1 Analyzer (EchoMRI, Houston, TX) in awake mice.

Mitochondrial isolation

Mitochondria were isolated from fresh triceps brachii muscles as reported previously (Frederick et al., 2015). Briefly, muscles were minced in ice-cold muscle homogenization buffer (100 mM KCl, 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM EDTA, and 1.8 mM ATP, pH 7.2), digested with 60 U/mL of protease from Bacillus licheniformis (Sigma, P5380), and further lysed with a Potter Elvehjem homogenizer for 10 minutes at 150 rpm. The homogenate was centrifuged twice at 720 xg for 5 minutes before supernatants were combined and spun at 10,000 xg for 20 minutes. The concentration of the final mitochondrial pellet was normalized in resuspension buffer (225 mM sucrose, 44 mM KH₂PO₄, 12.5 mM Mg-acetate, and 6 mM EDTA; pH 7.4) and immediately extracted for NAD measurement or used for respiration assays.

Mitochondrial respiration assays
Respiration of 0.15 mg of freshly isolated mitochondria was measured in MiRO5 respiration medium (110mM Sucrose, 20mM HEPES, 10mM KH$_2$PO$_4$, 20mM Taurine, 60mM K-lactobionate, 3mM MgCl$_2$•6H$_2$O, 0.5mM EGTA, 1g/L defatted BSA, pH 7.2) using a Clark-type electrode (Strathkelvin Instruments, North Lanarkshire, Scotland) at 37°C. All chemicals were obtained from Sigma. Sequential addition of 5 mM malate, followed by either 10 mM pyruvate or 20 µM palmitoyl carnitine, was provided as a substrate, and 4 µmol of ADP were added to stimulate maximal coupled respiration. Maximal uncoupled respiration was measured following addition of 2 µg/mL oligomycin from S. Diastatochromogenes and 4 µM carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP). Assays were ended by the addition of 5 µM antimycin A to cease respiration. Non-linear correlations with mitochondrial NAD content were calculated with Graphpad Prism 5 software.

Histology

Extensor Digitorum Longus (EDL) muscles were frozen in OCT and cryosectioned at 10 micron thickness. Sections were stained with hematoxilin and eosin (H+E) according to standard procedures. For immunofluorescence (IF), membranes and nuclei were counterstained with 1:100 dilutions of anti-laminin (L-9393, Sigma) and Hoechst 2223, respectively. Goat anti-rabbit secondary antibody conjugated to Alexa 488 (Jackson Immunoresearch, West Grove, PA) was diluted 1:500 and incubated for 30 minutes at 37°C. Sections were imaged using an EVOS FL Auto Cell Imaging System (Life Technologies). Analysis of fiber diameter and nuclear position was performed as described using SMASH software, as described previously (Smith and Barton, 2014).

Electron microscopy

EDL muscles that did not undergo the ex vivo physiology protocols were maintained at physiological length, fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at
room temperature and stored in fixative at 4°C until processed by the Electron Microscopy Resource Laboratory at University of Pennsylvania. Central cross-sectional and longitudinal muscle segments were post-fixed in 2% OsO₄ in cacodylate buffer and embedded. Sections were stained with lead citrate solutions and imaged using a Jeol-1010 Transmission electron microscope.

Bone analysis

Tibia bones were cleaned of connective tissue and fixed in 4% paraformaldehyde overnight at 4°C. Trabecular bone from each metaphysis and cortical bone from each midshaft was selected and scanned at 6 µm resolution using a µCT 35 system (Scanco Medical, Bruttisellen Switzerland). Physical parameters and three-dimensional reconstructions were calculated using associated proprietary software.

Exercise tolerance

Exercise tolerance was assessed using an Exer3-6 treadmill system with shock counter (Columbus Instruments, Columbus OH) running a step-wise accelerating protocol at 0° incline described previously (Frederick, 2015). Mice were habituated to the treadmill at 10 m/min for 15 min 24 hours before and fasted for 3 hours before the experiment. Exhaustion was defined as 50 cumulative stimuli. Lactate was measured from tail blood using a Lactate Pro handheld meter (Arkray, Japan).

Ex vivo muscle physiology

Muscle physiological analysis was performed on isolated EDL muscles using a 1200A Intact Muscle Test System equipped with Dynamic Muscle Control v.5.415 software (Aurora Scientific, ON, Canada) at the Muscle Physiology Assessment Core of the Pennsylvania Muscle Institute. EDL muscles were dissected and analyzed in constantly oxygenated Ringer's solution (100 mM NaCl, 4.7 mM KCl, 3.4 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM HEPES,
5.5 mM D-glucose) at 24°C. Maximal isometric twitch and tetanic contractions were obtained using a stimulation frequency of 2500 Hz for 0.2 msec or 120 Hz for 500 msec, respectively. Five minutes were allowed between tests to ensure muscle recovery. Specific force was determined by normalizing absolute force to muscle crosssectional area (CSA). CSA was calculated by dividing the muscle mass by the product of the muscle density coefficient (1.06 g/cm$^3$), muscle optimal length ($L_o$), and the fiber length coefficient (0.45 for EDL). Muscle stiffness was measured using a series of passive stretches (10 repetitions per cycle at 2Hz frequency) with increasing amplitude (5, 8, 10, 15, 20% of $L_o$) performed 5 minutes after the fatigue protocol. The Lissajous curves generated were used to calculate the force opposed by the muscle tissue to the lengthening work (Stedman et al., 1991; Syme, 1990).

**Immunoblotting**

Frozen tissues were lysed in lysis buffer (25 mM Tris pH 7.9, 100 mM KCl, 5 mM MgCl$_2$, 1% nonidet P-40, 10% glycerol) supplemented with protease inhibitor cocktail (Roche) using a motorized homogenizer. SDS-PAGE was performed on 20 µg of cleared lysate in Laemmli buffer loaded on 10% gels. Gels were transferred to PVDF membranes, blocked in 5% milk, and incubated with 1/1000 dilutions of anti-Nampt (Bethyl A300-372A) and anti-alpha tubulin (Abcam ab40742). Antibody binding was detected and imaged using a chemiluminescent substrate (Perkin Elmer) and Chemi-Doc system (Biorad).

**Transcriptome sequencing and analysis**

RNA was extracted from quad muscle using the RNeasy fibrous tissue kit (Qiagen) or Trizol reagent (Life Technologies) according to manufacturer protocols. Libraries were generated from 1 µg of total RNA using the TruSeq Stranded mRNA Library Prep kit according to the manufacturer’s instructions (Illumina). 100 bp single end sequencing was performed on the Illumina HiSeq 2000 at the Next Generation Sequencing Core at the University of Pennsylvania. Reads passing quality filters were trimmed with Cutadapt, mapped to the mouse genome (mm10).
with Tophat2, and read counts per gene were determined using HTseq (Anders et al., 2015; Martin, 2011; Trapnell et al., 2009). DEseq2 was used to perform differential expression analysis and to obtain normalized read counts after variance stabilizing transformation as previously described (Ghanem et al., 2015; Love et al., 2014). Normalized read counts were used to generate scatterplots and heatmaps. Genes with an adjusted p-value < 0.01 or <0.05 were considered significant in the mNKO and mNOE samples, respectively. Microarray data for mdx mice were obtained from GSE897 using GEO2R and represented in a heatmap generated from genes that were differentially expressed (+/- 2-fold change and p-value < 0.01) in both populations. Gene ontology analysis was performed using the online resource DAVID (Huang et al., 2009a, 2009b). Genes relating to muscle regeneration were described previously (Ryall et al., 2015). All sequencing data generated in this study has been deposited in the UCSC genome browser and GEO under the accession number GSE74570 (available at http://www.ncbi.nlm.nih.gov/geo/).

In vitro isotopic labeling

Isotope-labeling medium was prepared from phenol red-, sodium pyruvate-, sodium bicarbonate-free DMEM powder (Cellgro) supplemented with 3.7 g/ L sodium bicarbonate, 25 mM [1,2-13C]glucose (Cambridge Isotope Laboratories) and 4 mM glutamine.

C2C12 myoblasts (ATCC) were cultured in growth medium (20% fetal bovine serum, Gibco). Differentiation was induced by switching the cells to medium containing 2% equine serum (HyClone) supplemented with 1 µg/ml insulin (Sigma), which was changed daily for 3 days. Mature myotubes were treated with 100nM FK866 and either 10 or 100 µM NR. After 2 days, cells were switched to isotope-labeling medium for 12 hours, which was replaced 2 hours before harvesting cells, to ensure steady-state labeling (Munger et al., 2008). Metabolism was quenched and metabolites were extracted by aspirating media and immediately adding 2 mL 80:20 methanol:water on dry ice for 20 min. The resulting mixture was collected and centrifuged at 16,000 xg for 5 minutes. Insoluble pellets were re-extracted with 1 mL 80:20 methanol:water on
dry ice. The supernatants from two rounds of extraction were combined, dried under N₂, resuspended in 1 mL water per million cells, and subjected to mass spectrometric analysis. Cell number was determined with an automated cell counter (Invitrogen) and used for normalization of mass spectra.

**In vivo isotopic labeling**

Metabolites from 50 µl of whole blood were extracted by adding 200 µl ice cold methanol. After 20 minutes of incubation at -20°C and centrifuge at 16,000 xg for 10 min, the resulting supernatant was transferred and insoluble pellets were re-extracted with 1 mL 40:40:20 methanol:acetonitrile:water. The supernatants from two rounds of extraction were combined, dried under N₂, resuspended in 0.25 mL water. Metabolites were extracted from 50 mg of powderederized tissues by twice adding 1 ml of dry ice-cooled 80:20 methanol:water with 0.1 % formic acid, incubating for 5 minutes on dry ice, and centrifuging at 16,000 xg for 10 minutes. The supernatants from two rounds of extraction were combined, dried under N₂, resuspended in 1 mL water, and subjected to mass spectrometric analysis.

**LC-MS metabolomics**

Metabolites were analyzed within 12 hours of extraction by reversed-phase ion-pairing chromatography coupled with negative-mode electrospray-ionization high-resolution MS on a stand-alone orbitrap (Thermo)(Lu et al., 2010). NAM, NMN, NR, and NAD were analyzed by reversed-phase chromatography coupled with positive-mode electrospray-ionization on a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo). Liquid chromatography separation was achieved on a Poroshell 120 Bonus-RP column (2.1 mm ×150 mm, 3.7 µm particle size, Agilent). The total run time was 25 minutes with a flow rate of 50 µl/min from 0-12 minutes and 200µl/min from 12-25 minutes, applying a gradient of 0-70 % solvent B (acetonitrile) in 12 min, balanced by solvent A (98:2 water:acetonitrile, 10 mM amino acetate, 0.1 % acetic acid).
RESULTS

*Nampt-deficient muscle exhibits compromised energetics*

To test the idea that deficits in intramuscular NAD may directly contribute to functional decline in skeletal muscle, we first attempted to generate mice with cardiac and skeletal muscle-specific deletion of *Nampt* using a floxed allele in combination with the same muscle creatine kinase (*Ckmm*)-coupled Cre recombinase used previously to create Nampt transgenics (Frederick et al., 2015). To our surprise, only one female of the first 44 pups born to these crosses contained the homozygous deletion (1 in 4 expected), suggesting predominant embryonic lethality. Reasoning that the low-level activity of *ckmm*-Cre in the heart may be responsible for the lethality, we instead crossed the floxed allele to a mouse line carrying Cre under control of the myosin light chain-1f promoter, which deletes exclusively in skeletal muscle (Bothe et al., 2000). Pups born to these crosses, dubbed muscle-specific Nampt knockout (mNKO mice), appeared in the expected Mendelian ratios, were fertile, and had no obvious increase in mortality when observed as long as 10 months. Muscles from mNKO mice are deficient in Nampt (Figure 3.1A) and contain <15% of the normal intramuscular NAD concentration by 3 months of age in both genders (Figure 3.1B,C).

Muscle from mNKO mice exhibited a >60% decline in ATP content, clearly indicating energetic stress (Figure 3.1D). Since NAD is required by the three NAD-dependent dehydrogenases of the TCA cycle, we reasoned that mitochondrial ATP synthesis might be impaired by NAD restriction. Consistently, we found mitochondria isolated from mNKO skeletal muscle to be depleted of NAD to a similar extent as whole muscle (Figure 3.1E) and inherently limited in their ability to utilize pyruvate and palmitoyl carnitine for respiration (Figure 3.1F, 3.2A). This limitation was not reflected in whole body respiration or feeding behavior, however, and correlated with only a trend toward decreased locomotion during the less active daylight hours (Figure 3.1G,H, 3.2C-G). At night, when energetic demands are higher, mNKO display an
elevated respiratory exchange ratio (RER), indicating an increased reliance on carbohydrate oxidation compared to littermate controls (Figure 3.1I, 3.2G).

To test the ability of NAD-restricted muscle to perform under more demanding conditions, we conducted treadmill exercise tolerance tests. Surprisingly, mNKO mice aged 3 months appeared to fatigue at the same rate as littermate controls (Figure 3.1J). Nampt deficient mice were noted to be slightly smaller at this young adult age, apparently due to an overall reduction in lean mass (Figure 3.1K). However, this difference was not reflected in the weights of the major hindlimb muscles used for running (Figure 3.1L). When contractile function was tested ex vivo, we found no change in the ability of mNKO muscles to generate force during short twitches, yet significant declines in the ability of these muscles to sustain maximal tetanic contractions (Figure 3.1M, N), suggesting that the primary functional impact of NAD loss may be muscle weakness. A clinical hallmark of muscle weakness is decreased density and altered architecture of the bones to which muscles routinely apply tensile force. Upon examination of the cortical bone of the tibia by microcomputerized tomography (microCT), we found no significant decrease in bone mineral density, nor in the number or spacing of trabecular plates from 3-month-old mice (Figure 3.4C-E). However, decreased trabecular thickness, thought to contribute to age-related bone loss (Weinstein and Hutson, 1987), was already apparent in mNKO tibias by this young age and contributed to visibly rod-like trabecular morphology (Figure 3.4F,G).

*mNKO mice develop myonecrosis and progressive loss of muscle function*

We next investigated whether the observed muscle weakness in mNKO mice might have morphological correlates. Indeed, mutant hindlimb muscles showed an overall decrease in average fiber size accompanied by wider variability in this parameter, development of muscle stiffness, and a dramatic 8-fold increase in the appearance of centrally nucleated fibers, indicative of recent regeneration (Figure 3.3A,B, 3.4A). The compromised membranes of myonecrotic
patches that appeared in histological sections were confirmed by intraperitoneal administration of Evans blue dye (EBD), which visibly accumulated in the mNKO hindlimbs and localized to a minority of discrete fibers (Figure 3.3C). Thus, the energetic deficit in mNKO muscle parallels the loss and regeneration of individual fibers.

Mouse models of severe muscular dystrophy exhibit disease progression which correlates with diminished proliferative potential of muscle progenitor cells (Lu et al., 2014; Sacco et al., 2010). To determine if the mNKO myopathy similarly worsened over time, we monitored body weight and endurance. Though equal tibia lengths indicated that mutants were skeletally full grown (Figure 3.4B), the slight decrease in lean mass observed in 3 month old male knockouts represented the beginning of a plateau in this parameter, driving a divergence in body weight as control littermates continued to gain weight (Figure 3.3D,E). By 7 months of age, the difference in muscle mass was apparent in the hindlimbs (Figure 3.3F). Females, which normally develop lean mass at a slower rate, exhibited no change in body weight. Upon exercise challenge, we found that 7-month-old mNKO mice could no longer maintain the treadmill performance of littermate controls (Figure 3.3G). Consistently, ex vivo twitch and tetanic force generation became severely limited by this age (Figure 3.3H,I), while immune cells appeared to increasingly infiltrate the endomysial space (Figure 3.3J).

To further dissect the sequence of events leading to NAD-deficient myopathy, we examined electron micrographs of 7-month-old muscle. We observed a clear pattern of discrete necrotic fibers in mNKO mice, often juxtaposed with fibers displaying remarkably normal sarcomeric and mitochondrial morphologies (Figure 3.3K). A minority of intermediate fibers exhibited swollen sarcoplasmic reticula, often associated with altered calcium homeostasis (Zhao et al., 2010), while others appeared only as remnants in the process of being cleared by multi-locular phagocytic immune cells (Figure 3.3K, bottom right). Muscle wasting phenotypes have been reported following genetic activation of NF-κB and Hif-1α transcription factors (Cai et al., 2004; Mason et al., 2004), whose activity might be de-repressed by loss of NAD-dependent Sirt6
or Sirt1 (Tennen and Chua, 2011; Yeung et al., 2004). To examine specific signaling pathways that might be linked to muscle wasting in mNKO mice, we performed whole transcriptome sequencing of mRNA isolated from quadriceps muscles. We were surprised to find fully 33% of detectable genes to be significantly altered in mNKO muscle, including those encoding 267 established transcription factors (Figure 3.5A,B). At this stage in the phenotypic progression, we were not able to isolate the effects of any one transcriptional regulator. Therefore, we partitioned all detected genes into four distinct groups using k-means clustering and found significant enrichment of genes relating to muscle injury, inflammation, and immune response (cluster 4), while the most down-regulated cluster (3) corresponded to metabolic processes (Figure 3.5C).

Unexpectedly, muscle-specific ubiquitin ligases MuRF1 (Trim63) and Atrogin1 (Fbxo32), associated with proteasome-dependent muscle wasting, were either unchanged or downregulated (Figure 3.5A,D). Nonetheless, expression of genes specific to muscle regeneration were highly enriched in the knockout (Figure 3.5E). Also of interest, the expression of p16ink4a (Cdkn2a), a gene strongly correlated with senescence and observed to increase in geriatric muscle satellite cells (Sousa-Victor et al., 2014), was dramatically up-regulated in the mutant, while additional overlap with age-related transcriptional changes was mild (Figure 3.5F). Moreover, we observed a striking overlap of differentially expressed genes with the mdx model of Duchene’s Muscular Dystrophy (DMD)(Haslett et al., 2005), highlighting the extent of the muscle injury (Figure 3.5F).

**Nicotinamide riboside functionally and morphologically restores NAD-deficient muscle**

Metabolomic studies of NAD restriction in cancer cells and erythrocytes have shown that inhibition of glycolysis at the level of NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is primarily responsible for diminished ATP production (Hikosaka et al., 2014; Tan et al., 2013). Consistent with these reports, our metabolomic analysis indicated that substrates of GAPDH, as well as upstream intermediates of the pentose phosphate pathway
(PPP), were significantly elevated in mNKO muscle (Figure 3.6A,B). However, intermediates in the distal part of the glycolysis pathway and the TCA cycle were not detectably decreased \textit{in vivo}, suggesting that mitochondrial function would not be limited by the supply of pyruvate. We also noted that Nampt deletion did not result in the accumulation of its NAM substrate, but rather in the appearance of methyl-NAM, an alternative fate for NAM equivalents.

The simple molecular basis of the defect in Nampt-deficient muscle led us to test whether the mNKO phenotypes might be reversed by augmenting the NAD salvage pathway via NAM-containing metabolites capable of entering the pathway upstream of Nampt. We first modeled this idea \textit{in vitro} using differentiated C2C12 myotubes in the presence of the specific Nampt inhibitor FK866. The resulting metabolomic profile paralleled that of mNKO muscle, but included decreases in distal glycolytic and TCA cycle intermediates, consistent with a more severe block in GAPDH activity (Figure 3.6C). Using a [1,2-$^{13}$C]-glucose tracer, we confirmed that the labeling patterns of fructose-1,6-bisphosphate (FBP) and ribose-5-phosphate (R5P) reflected elevated carbon flux into the non-oxidative arm of the PPP, an alternative pathway for metabolites upstream of GAPDH (Figure 3.6D,E). Importantly, these patterns were completely reversed following addition of 10-100 µM nicotinamide riboside (NR) to the culture media (Figure 3.6C,E), indicating that NR can be directly utilized by myotubes to effectively bypass the requirement for Nampt activity.

To test whether NR can bypass Nampt deficiency \textit{in vivo}, we dissolved the compound in the drinking water to deliver an effective daily dose of \~{}400 mg/kg. Mice aged 5-6 months received the treatment continuously for 6 weeks. Intriguingly, this intervention appeared to completely prevent the development of exercise intolerance observed in 7-month-old mNKO mice (Figure 3.7A). Histological analysis confirmed that NR treatment induced muscle fiber remodeling in the mutant, partially restoring fiber diameter without affecting the degree of central nucleation (Figure 3.7F). Moreover, while exercised mNKO mice displayed a dramatic elevation of plasma lactate, the concentration was restored to near control levels in the NR-treated group (Figure
This is consistent with elevated glycolysis compensating for a deficit in mitochondrial function in the untreated mNKO mice. Accordingly, we found that NR ameliorated the metabolic dysfunction of mitochondria isolated from mNKO muscle, completely restoring the maximal coupled respiration on pyruvate in a manner that correlated with the NAD content of the matrix (Figure 3.7B-D). While muscle ATP largely rebounded as a consequence, this effect was not mirrored by whole muscle NAD levels, which showed only a small trend toward an increase in NR-treated mutants (Figure 3.7G,H).

We next examined the ability of NR to reverse established muscular dysfunction by beginning a 6-week intervention in male knockout mice at 5.5 months of age, when loss of lean mass and endurance was already apparent. Consistent with rapid restoration of metabolic flux, mNKO mice experienced a complete restoration of exercise capacity after only one week, which persisted for the entire 6-week duration of the treatment (Figure 3.8C-F). We further demonstrated the near complete restoration of force generated by isolated mNKO muscles, as well as normalization of mass in all major hindlimb muscles, with no discernable effect of NR on littermate controls (Figure 3.8G-I). Whereas ATP levels in mNKO mice were nearly restored to those of wild type controls after NR treatment, we again found that NAD content of whole NR-treated mNKO muscle remained severely depleted, with only a trend toward improvement when compared to the untreated knockouts (Figure 3.8A,B).

Reasoning that poor bioavailability might be responsible for the weak impact of NR on steady state muscle NAD levels, we designed an isotope-labeled NR tracer, with a single $^{13}$C and a single deuterium on the nicotinamide and ribose moieties, respectively, to elucidate the location and molecular form of its systemic distribution (Figure 3.7I). Direct incorporation of the tracer into NAD yields M+2 NAD, whereas breakdown and resynthesis by the salvage pathway of any cell yields M+1 NAD (Figure 3.7J). We found that 100 minutes after oral administration, the labeled NR was readily detectable in liver, but not in skeletal muscle (Figure 3.7K,L). Furthermore, a previously unappreciated natural abundance of NR was suppressed in the mutant, suggesting the
existence of an equilibrium with NMN, perhaps mediated by 5'-nucleotidases (Grozio et al., 2013). A minute amount of doubly labeled NAD was observed in muscle, indicating that direct contribution of NR to the muscle is possible and likely contributes to the small increases in NAD content that we observed. However, the vast majority of orally administered NR is processed by the liver and other tissues before entering the NAD salvage pathway of muscle in the form of NAM or NMN (Figure 3.7M,N), indicating a complex route of distribution.

Gain of Nampt function maintains exercise capacity into old age

Following our earlier studies indicating little impact of Nampt overexpression in cardiac and skeletal muscle of young mice (Frederick et al., 2015), we decided to examine the effect of lifelong muscle-specific Nampt transgene expression (mNTG mice) near the end of the expected mouse lifespan at 24 months of age (Figure 3.9A). When compared to young adult cohorts, aged controls exhibited reductions in intramuscular NAD of approximately one third, in line with previous reports (Figure 3.9B). While transgenic mice also exhibited a decline with age, the higher starting point rendered intramuscular NAD levels in aged mNTG mice indistinguishable from that of young controls. Interestingly, aged mNTG mice exhibit a moderate reduction in body weight (Figure 3.9C) and marked improvement in exercise capacity, closer to the performance of young mice than to that of the age-matched controls (Figure 3.9D, E). To determine whether the enhanced exercise capacity was secondary to the induction of an NAD-dependent transcriptional program in muscle, we performed whole-transcriptome sequencing of mRNA from quadriceps muscle. To our surprise, only 18 of the more than 15,000 detected genes were significantly altered by the transgene when compared to age-matched 24-month-old controls, even using relaxed selection criteria (Figure 3.9F). From these, no obvious candidates for regulation of oxidative metabolism emerged. Furthermore, the age-related changes in gene expression in the mNTG generally mirrored those of controls, significantly overlapping in 87 instances and opposing in none (Figure 3.9G,H). The lack of strong transcriptional changes favors the model
that Nampt overexpression prevents age-related decline in muscle function through a direct metabolic mechanism: by facilitating glycolytic and TCA cycle flux.

DISCUSSION

To better understand the physiological and therapeutic implications of altering NAD metabolism within skeletal muscle, we created a model of primary pyridine nucleotide deficiency using a floxed allele of Nampt. Our finding that the NAD content of muscle was decreased by ~85% confirmed the prevailing view that the salvage route of NAD synthesis from NAM sustains the vast majority of the NAD utilized by this tissue. Neither the natural abundance of circulating NMN, nor extracellular Nampt (eNampt) (Revollo et al., 2007a) appear sufficient to alleviate the resulting pathology, indicating that muscle NAD metabolism is largely an isolated system. Nonetheless, our observation that mNKO mice appear grossly unaffected for several weeks post-weaning illustrates the incredible metabolic flexibility of skeletal muscle and supports the proposition that intramuscular NAD of young wildtype mice is maintained at a level far in excess of that absolutely required for muscle function.

Given the essential nature of NAD in bioenergetics, there must exist a threshold below which cells depleted of this metabolite experience an energetic crisis. Despite the normal structural features of many fibers in mNKO mice, the loss of energy charge from adenine nucleotides in their muscles suggest that they are approaching this threshold. Pharmacological inhibition of Nampt in cultured tumor cells has been found to restrict glycolytic flux at the level of GAPDH, the only NAD-dependent enzyme in this pathway, creating a signature increase in the prevalence of upstream metabolites and depletion of downstream metabolites (Tan et al., 2013). We observed a nearly identical pattern in cultured myotubes treated with FK866. Accumulation of upstream metabolites was also apparent \textit{in vivo}, supporting the proposal of Tan and colleagues.
that measuring proximal glycolytic intermediates as biomarkers of severe NAD restriction in biopsied tissue samples may have clinical utility (Tan et al., 2013). However, our observations that downstream metabolites were not depleted in muscles of mNKO mice and that running the mNKO animals to exhaustion still caused lactic acidosis suggest a more moderate inhibition of GAPDH, perhaps compensated by mass action to maintain glycolytic flux. These findings strongly implicate the observed mitochondrial dysfunction as the likely cause of ATP depletion observed in mNKO mice. It is noteworthy that a previously reported model of skeletal muscle cytochrome c oxidase deficiency was found to survive as long as four months, despite near complete deficiency in mitochondrial function (Diaz, 2005), indicating that glycolysis alone can support a substantial portion of the energetic burden in youthful muscle. Importantly, means of restoring mitochondrial function were subsequently found to reverse the myopathy and extend the survival of these animals (Wenz et al., 2008).

Our finding that a single week of NR supplementation was sufficient to dramatically restore exercise capacity in mNKO mice points to restoration of muscle metabolic flux as the likely mechanism. Indeed, the remodeling required to increase oxidative capacity in muscle typically requires several weeks or months. While NR could conceivably contribute to the endurance phenotype through a variety of effects in other tissues, our ex vivo experiments favor a model that is at least partially muscle fiber-autonomous. The persistent restoration of peak twitch and tetanic force in isolated muscles indicates that the effect is not strictly dependent on the neuromuscular junction or the acute supply of circulating metabolic substrates. Similarly, the restored oxidative capacity of mitochondria persists post-isolation and appears to correlate with the increases in the NAD content of the organelles, suggesting a mechanistic link. In light of its potent phenotypic effects in mNKO mice, we were nonetheless surprised to find that NR exerts only subtle influences on the steady state concentration of NAD in whole muscle or mitochondria. The simplest interpretation of these results is that the effects of NAD replenishment are more specifically localized, perhaps disproportionately benefiting the minority of fibers with the lowest NAD concentrations. Our findings in NR-treated mNKO mice may also reflect the existence of
nuclear or cytoplasmic NAD microdomains, which would allow the activity of NAD-dependent enzymes to be uncoupled from the absolute size of the tissue NAD pool (Grubisha et al., 2005). In this model, metabolic flux is supported by close juxtaposition of the enzymes that synthesize and consume NR-derived NAD. Thus, functional NAD turnover would be increased to a greater extent than its steady state concentration. Another consideration is that NAD can influence metabolism secondary to its effects on calcium homeostasis. Indeed, the supply of calcium has been suggested to be as essential to the mitochondrial dehydrogenases as that of NAD (Cárdenas et al., 2010), and may serve as a secondary messenger to amplify the effects of NAD replenishment. Additionally, our results leave open the possibility that some of the functional improvements in differentiated muscle are secondary to effects in other cell types. For example, if NR enhances the myogenic potential of resident satellite cells or suppresses the activity of resident immune cells via local elevations in NAD, these changes would be nearly undetectable in whole tissue homogenates. Similar indirect activities may help to explain how oral NR administration clearly mitigates the severity of insults to a growing list of tissues in which robust NAD decrements have not been demonstrated before treatment (Brown et al., 2014; Cerutti et al., 2014b; Khan et al., 2014a; Xu et al., 2015).

Though isolated disturbances in NAD synthesis are uncommon in nature, the impairment of ATP production observed in the mNKO mice is a characteristic feature of metabolic myopathies, which include numerous muscle wasting syndromes stemming from inborn errors of metabolism and successfully modeled in mice (Cerutti et al., 2014b; Diaz, 2005; Graham et al., 1997). A key similarity between these models and the phenotypes of mNKO mice is the progressive decline in muscle contractile function over weeks or months, accompanied by altered myofibrillar morphology. Unlike most myopathies, however, the death of mNKO muscle fibers occurs in the absence of any upregulation of the catabolic ubiquitin ligases Atrogin1 or MuRF1, implicating a largely unregulated mechanism of cell death. Rather, this pattern mirrors the well-documented mechanism of poly(ADP) ribose polymerase-mediated cell death, in which rapid NAD consumption restricts intracellular ATP to a level below that required by the apoptotic
program, instead promoting necrosis (Ha and Snyder, 1999). Our histology and electron microscopy provide evidence consistent with this mechanism. Transcriptionally, there is a striking resemblance between mNKO muscle and that of the well-characterized mdx model of DMD. This observation was unexpected, given that the former defect is strictly metabolic and the latter strictly structural, and especially compelling in light of reports that mdx muscle contains less NAD than that of age-matched controls, and that NAD supplementation appears to ameliorate an additional laminopathy modeled in zebrafish (Chalkiadaki et al., 2014; Goody et al., 2012). Thus, our finding that muscle fiber atrophy can be derived solely from disrupted NAD salvage raises the intriguing possibility that NAD loss contributes to the etiology of at least a subset of established muscular dystrophies.

An important goal of this work was to determine whether primary NAD deficiency could mimic age-associated physiological decline in muscle function. We were intrigued to find the expression of Cdkn2a, encoding the mitotic checkpoint inhibitor commonly known as p16ink4a, dramatically upregulated in mNKO muscle, since this biomarker of senescence is closely associated with aging in many tissues, including muscle (Krishnamurthy et al., 2004). Yet, while mNKO mice clearly exhibited progressive weakness and loss of both endurance and bone structure, the decline in NAD was more severe than has been observed for normal aging. Furthermore, the transcriptional signature predominately reflected the muscle regeneration and immune infiltration, making it difficult to assess whether more subtle features of aging were emergent. In this regard, the overexpression model offers important insights. Complementary to our results in mNKO mice, preventing the natural decline of intramuscular NAD in 24-month-old mNTG mice significantly improved exercise capacity as compared to littermate controls. Interestingly, transcriptome analysis indicated that this improvement is not secondary to any major changes in gene expression, suggesting a model in which metabolic flux and/or NAD-dependent post-translational modifications play a major role. The implication that the modest age-related decline in NAD can have functional consequences is in stark contrast to the ability of young mice to initially tolerate a much more severe depletion, and suggests that one or more
additional factors may aggregate over time to exacerbate the dependence of muscle function on internal NAD stores. Alternatively, the aggregate decline in NAD with age may be driven by a subset of fibers with more severe depletion. The mechanisms by which Nampt overexpression can restore function in aged, and perhaps dystrophic muscle will be an important area for future investigations. The mNKO model may further be employed to resolve the efficacy of new therapeutics designed to boost muscle NAD content. Overall, our results clearly indicate that Nampt expression is critical to the maintenance of both the mass and contractile function of skeletal muscle, lending support to the idea that loss of NAD homeostasis may contribute to genetic and age-related causes of physiological decline in this tissue.
FIGURES AND TABLES

Table 3.1. *Concentrations of redox-sensitive metabolites in quadriceps muscle of 24-month-old mice.* Concentrations presented as mean nmol/mg tissue (SEM). β-HB, β-hydroxybutyrate. *p<0.05 compared to control, #p<0.05 compared to NC by one-way ANOVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Observations</th>
<th>[Lactate]</th>
<th>[Pyruvate]</th>
<th>[β-HB]</th>
<th>[Acetoacetate]</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td>6</td>
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<td>0.056 (0.005)</td>
<td>0.105 (0.015)</td>
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<tr>
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<td>0.069 (0.008)</td>
<td>0.118 (0.014)</td>
</tr>
<tr>
<td>NNC</td>
<td>4</td>
<td>8.4 (1.8)</td>
<td>0.130 (0.017)*#</td>
<td>0.053 (0.007)</td>
<td>0.109 (0.020)</td>
</tr>
</tbody>
</table>
Figure 3.1. *Characterization of mice deficient for Nampt in skeletal muscle*

A, Expression of Nampt protein in hindlimb muscles of skeletal muscle-specific Nampt knockout (mNKO) mice compared to floxed littermates. Gastrocnemius (gast.), tibialis anterior (TA), and quadriceps (quad) of 3-month-old male mice represented.
B, Intramuscular NAD content of hindlimb muscles from female (n=8-10) and

C, Male (n=4-6) mice assessed at 3 months of age.

D, Intramuscular ATP content (n=4-6).

E, NAD content of mitochondria isolated from triceps brachii muscle (n=4-5).

F, Respiratory capacity of isolated muscle mitochondria sequentially provided with pyruvate (pyr),
malate (mal), and ADP, followed by complex V inhibitor oligomycin (oligo), uncoupler (FCCP),
finally complex III inhibitor antimycin A (AntA) (n=4-6).

G, Mean oxygen consumption (VO₂) normalized to lean body mass (n=7-9) and

H, Voluntary ambulatory activity during light and dark hours (n=8-10).

I, 24-hour time course of respiratory exchange ratio (RER)(n=7-9, *p<0.05).

J, Treadmill running performance (n=6 at baseline). Dashed line indicates exhaustion threshold.

K, Body composition assessed by NMR (n=7-9).

L, Mass of isolated hindlimb muscles (n=6-10).

M, Normalized twitch force generated by isolated EDL muscle. Dashed lines indicated error bars.
Inset: maximum force generated per subject (n=4-6).

N, Normalized tetanic force generated by isolated EDL muscle. Dashed lines indicated error bars.
Inset: maximum force generated per subject (n=4-6). Error bars represent SEM. Significance was
determined by Student’s t-test (*p<0.05, **p<0.01, ***p<0.001). Mice were 3-month-old males
unless otherwise indicated.
Figure 3.2 Effects of muscle Nampt deficiency on mitochondrial and whole body respiration and feeding (related to Figure 3.1)

A, Respiratory capacity of isolated muscle mitochondria sequentially provided with palmitoyl carnitine (PC), malate (Mal), and ADP substrates, followed by oligomycin (oligo), FCCP, and antimycin A (AntA)(n=4-6).

B, 24-hour time course of VO₂ normalized to lean mass.
C, 24-hour time course of VCO₂ normalized to lean mass.

D, VCO₂ normalized to lean mass during light and dark hours.

E, 24-hour time course of food intake normalized to lean mass.

F, Food intake normalized to lean mass during light and dark hours.

G, Mean RER during light and dark hours. Error bars represent SEM. Significance was determined by Student’s t-test (*p<0.05, **p<0.01). Mice were 3-month-old males (n=8-10 unless otherwise indicated).
Figure 3.3. Loss of salvage NAD synthesis results in progressive muscle degeneration

A, Histological sections of EDL muscle from 3-month-old mice stained with H+E or laminin and DAPI to highlight fiber morphology. The scale bar represents 50 µm.
B, Distribution and descriptive statistics of minimum Feret diameters of EDL fibers from 3-month-old mice. Inset: proportion of centrally nucleated fibers (CNF). At least 2.4x10^3 fibers from 3 individuals per group were considered.

C, Representative hindlimb musculature of 3-month-old mice 16 hours after intraperitoneal injection of Evans Blue Dye (EBD). Inset: Histological sections of whole TA muscle stained with laminin and exhibiting fluorescence of EBD.

D, Growth curves from weaning to adulthood (n=7-12 males, 4-15 females).

E, Body composition assessed by NMR at 7 months of age (n=5-10).

F, Mass of isolated male hindlimb muscles at 7 months of age (n=6-10).

G, Treadmill running performance examined at 7 months of age. Dashed line indicates exhaustion threshold (n=6).

H, Normalized twitch force generated by isolated EDL muscle. Dashed lines indicated error bars. Inset: maximum force generated per subject (n=5-10).

I, Normalized tetanic force generated by isolated EDL muscle. Dashed lines indicated error bars. Inset: maximum force generated per subject (n=5-10).

J, Histological sections of EDL muscle from 7-month-old mice stained with H+E or laminin and DAPI. The scale bar represents 50 µm.

K, Electron micrographs of EDL muscle taken from female mice aged 7 months. Top left: adjacent fibers in a control muscle. Top middle and top right: A disordered necrotic fiber (yellow asterisk) next to a healthy fiber in an mNKO muscle. Bottom left: Grossly normal fiber morphology in an mNKO muscle. Bottom middle: Pre-necrotic fiber morphology showing swollen sarcoplasmic reticulum (yellow arrows) in an mNKO muscle. Bottom right: Phagocytic cell engulfing debris of a
necrotic mNKO fiber (yellow asterisk). Error bars represented SEM. Significance was determined by Student's t-test (*p<0.05,***p<0.001). Mice were male unless otherwise indicated.
Figure 3.4 Musculoskeletal features of mNKO mice (related to Figure 3.3)
A, Resistance to stretch in EDL muscle isolated from 3-month-old mice.

B, Length of tibia bones indicates normal skeletal growth.

C, Relative trabecular bone volume (VOX BV/TV),

D, Number (TB.N),

E, Spacing (TB.Sp), and

F, thickness (TB.Th) of trabecular plates in metaphysis of tibia bones (n=6-8). Red circles indicate the representative subjects pictured in G.

G, Representative three-dimensional reconstructions of trabecular bone. Scale bars represent 0.5 mm. Error bars represent SEM. Significance was determined by Student’s t-test (*p<0.05,**p<0.01). Mice were male aged 3 months.
Figure 3.5. Muscle Nampt deficiency induces pro-inflammatory and regenerative transcriptional programs

A, Correlation plot of RNAseq gene expression profiling in quadriceps muscle from 7-month-old mice (n=4). The Nampt and Cdkn2a transcripts are indicated. Red dots indicate significant differences in expression between groups.

B, Heatmap of gene expression in mNKO muscle with each genes assigned to one of four groups according to k-means clustering. Colors indicate fold change (FC) normalized to group means.

C, Selected gene ontology of differentially regulated clusters in B.

D, Coverage of sequencing reads (UCSC browser) for transcripts of the muscle-specific ubiquitin ligases Trim63 and Fbxo32.

E, Heat map of differentially expressed genes relating to muscle regeneration in mNKO.
F, Heatmaps of commonly differentially expressed genes. Left: Control mice aged 24 months vs. 4 months compared to mNKO vs. age-matched controls. Right: The *mdx* model of muscular dystrophy vs. age-matched wildtype controls compared to mNKO vs. age-matched controls.
**Figure 3.6. NAD restriction limits both glycolysis and OXPHOS**

A, Heatmap indicating relative abundance of metabolites detected in 7-month-old male quadricep muscle, subdivided by metabolic pathway (n=5-10).

B, Schematic representation of metabolomic analysis in A. Colored metabolites were differentially detected to varying degrees in mNKO muscle while unchanged metabolites did not reach statistical significance. Location of the oxidative pentose phosphate pathway (oxPPP) and the catalytic activity of Nampt and GAPDH are indicated.
C, Heatmap indicating relative abundance of metabolites detected in C2C12 myotubes treated with 10 nM FK866 and 100 µM NR, subdivided by metabolic pathway. Grey boxes represent undetected metabolites (n=3).

D, Schematic representation of $^{13}$C isotope incorporation (red circles) into intermediates of the glycolysis and pentose phosphate pathways following addition of [1,2-$^{13}$C]-glucose to culture cells. Distinct labeling patterns due to oxPPP activity and Aldolase reversal are indicated.

E, Isotope labeling of metabolites in C2C12 myotubes after 12 hours of exposure to [1,2-$^{13}$C]-glucose. Labeling patterns of fructose-1,6-bisphosphate (FBP) and ribose-5-phosphate (R5P) are represented (n=2-3). Error bars represented SEM. Significance was determined by one-way ANOVA with Tukey post-hoc test (*p<0.05, ***p<0.001).
A, Treadmill running performance of 7-month-old mice provided with unsupplemented drinking water (Veh) or NR-supplemented drinking water for 6 weeks (n=4-8).

B, Respiratory capacity of muscle mitochondria provided with excess palmitoyl carnitine or pyruvate.

C, Excess pyruvate (n=4-6, significant changes relative to mNKO Veh).

Figure 3.7. NR ameliorates deficits in mNKO mitochondrial function and fiber morphology
D, Relationship between oxygen consumption and NAD content of mitochondria isolated from skeletal muscle (n=4-6). The line indicates a rectangular hyperbolic least squares regression and associated correlation coefficient.

E, Concentration of blood lactate at baseline and at the point of exercise exhaustion (n=4-8).

F, Distribution and descriptive statistics of minimum Feret diameters of EDL fibers. Inset: proportion of centrally nucleated fibers (CNF). At least $2.0 \times 10^3$ fibers from 3 individuals per group were considered.

G, Intramuscular NAD content (n=4-7) and

H, Intramuscular ATP content (n=4-7) in quadriceps muscle following NR treatment.

I, Molecular structure of the NR$^{M+2}$ mass isotopologue. Green and red arrows indicate locations of $^{13}$C and deuterium labels, respectively.

J, Schematic of proposed NR$^{M+2}$ entry into the NAD salvage pathway (dashed lines, ATP not shown) and the subsequent separation of the isotopes on ribose and NAM moieties. Basal metabolic redox processes do not affect the arrangement of the labels.

K, Detection of NR isotopologues in quadriceps muscle (n=3) and

L, Liver tissue 100 minutes after oral gavage with 200 mg/kg NR$^{M+2}$. Some isotopologues were not detected (n.d.) in muscle (n=3).

M, Incorporation pattern of heavy isotopes into intermediates of the NAD salvage pathway in skeletal muscle (n=3) and

N, Liver tissue 100 minutes after oral gavage with 200 mg/kg NR$^{M+2}$ (n=3). Error bars represented SEM. Significance was determined by one-way ANOVA with Tukey post-hoc test (ns, not significant, *p<0.05, **p<0.01). All mice were female aged 7 months.
Figure 3.8. NR reverses deficits in muscle mass, strength, and exercise capacity of mNKO mice.
A, Intramuscular NAD content (n=4-6) and

B, Intramuscular ATP content assessed in quadriceps after 6 weeks of treatment (n=4-6).

C, Treadmill performance of 5-month-old mice before beginning NR administration and

D, after 7 days of treatment (n=5-6 at baseline).

E, Change in running distance of individuals at the point of exhaustion across the first 7 days of the NR treatment period.

F, Treadmill performance after 5 weeks of NR treatment (n=5).

G, Mass of hindlimb muscles isolated after 6 weeks of NR administration (n=5-6).

H, Normalized twitch force generated by isolated EDL muscle. Dashed lines indicated error bars. Inset: maximum force generated per subject (n=5-6).

I, Normalized tetanic force generated by isolated EDL muscle. Dashed lines indicated error bars. Inset: maximum force generated per subject (n=5-6). Error bars represented SEM. Significance was determined by one-way ANOVA with Tukey post-hoc test (ns, not significant, *p<0.05, ***p<0.001). All mice were male aged 7 months.
Figure 3.9. *Lifelong elevation of muscle Nampt expression increases exercise capacity of aged mice*

A, Expression of exogenous Nampt protein persists in muscle until 24 months of age.
B, Age-related alterations in intramuscular NAD content assessed in gastrocnemius muscle of 4-month-old (4 mo) and 24-month-old (24 mo) mice with one (mNTG$^{het}$) or two (mNTG) copies of the Nampt transgene (n=4-11).

C, Body weights of old mice were moderately affected by the transgene (n=7-14).

D, Treadmill performance in 24-month-old mice. Horizontal dashed line indicates the exhaustion threshold. The blue dashed line indicates the performance of young control littermates. Inset: time elapsed at the point of exhaustion (n=7-14).

E, Histogram and descriptive statistics of stimuli received by group and binned by running distance at the point of exhaustion, as described in D. Vertical dashed lines indicate group means. The blue arrow indicates the mean running distance of young control littermates.

F, Correlation plot representing RNAseq gene expression profiling in quadricep muscle in 24-month-old mice (n=3-5). Labeled red dots indicate significant differences in expression (Padj, adjusted p-value) with associated gene names indicated.

G, Heatmap of relative gene expression reveals negligible impact of sustained NAD salvage on transcriptional programs of muscle with age. Genes in mNTG are presented based on significant differences in expression between controls aged 4 vs. 24 months.

H, Overlap in differentially regulated genes of control and mNTG muscle between 4 and 24 months of age. There were no significantly opposing transcripts between the genotypes. Young control data are reproduced from previously published results (Frederick et al., 2015). Error bars represented SEM. Significance was determined by one-way ANOVA with Tukey post-hoc test (ns, not significant, *p<0.05,**p<0.001). Mice were male of respective ages.
CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

“In the muscle, nature has produced a machine, so startling and at the same time so perfect, that the explanation of its mechanism could give satisfaction not only to the searching mind, but also promise a rich harvest to the technical progress of mankind”

-Otto Fritz Meyerhof
Summary of Key Findings

The core finding of these studies is that expression of the Nampt enzyme is necessary, but not sufficient to promote maximal oxidative function of muscle in young adult mice. The requirement for Nampt activity in mature skeletal muscle is clear, if not as immediate as originally anticipated, and closely tied to ATP production. Indeed, it can only be inferred that Nampt is more essential to cardiac muscle, containing myocytes with the highest ATP turnover, since embryos carrying both the floxed allele and Ckmm-Cre did not survive until birth. The mNKO model may be most surprising for its ability to perform stereotypical behaviors with relative normalcy for months, including ambulation, feeding, and reproduction, despite a massively depleted intramuscular NAD pool. The finding that young mNTG mice are similarly unaffected by the increased synthesis of intramuscular NAD suggests that youthful muscle readily accommodates as much as 50% variability in the steady state level of this metabolite for extended periods, with few functional consequences. However, another major finding of this work is that aged muscle appears to be significantly more sensitive to perturbations in the NAD pool, such that preventing even a small loss of the metabolite serves to maintain whole body exercise performance (summarized in Figure 4.1). This sensitivity, as well as the progressive development of the mNKO myopathy, may indicate that the impact of NAD deficiency on muscle is an integration of lost signaling events over time. Thus, while transient loss of NAD is well tolerated, the chronic condition becomes increasingly deleterious. Impressively, insofar as exercise capacity reflects whole body metabolic health, ours is only the second example of a muscle-specific transgene demonstrated to improve the healthspan of a mammal (Wenz et al., 2009). Collectively, these studies serve as a proof-of-principle that NAD-boosting therapeutics may have utility for slowing the rate of age-related physiological decline.

In recent years, the regulation of Nampt activity has fit primarily into hypothetical models in which the enzyme serves to amplify or relay signals initiated by energetic stress (Cantó et al., 2010; Fulco et al., 2008). These models informed our prediction that Nampt overexpression might
influence muscle by shrinking the NAM pool or increasing the NAD/NADH ratio. After finding no evidence for either of these effects in young mNTG muscle, we are forced to consider an alternative model, in which Nampt serves primarily to maintain NAD homeostasis within margins necessary for basal metabolic processes to generate ATP.

The mNTG and mNKO mice provide several lines of evidence in support of this model. First, it is clear that skeletal muscles of mice aged 4-12 weeks naturally produce at least five-fold more NAD than is absolutely required to maintain muscle growth and exercise performance (Figure 3.1), perhaps explaining why enhanced NAD synthesis did not affect these parameters in young adult mice (Figure 2.3). Second, sustained decreases in the local NAD pool clearly limit muscle mass and contractile performance (Figure 3.3), indicating that Nampt controls NAD homeostasis under normal conditions at a level that is essential to the maintenance of muscle tissue. Third, the muscle mitochondrial NAD pool is sensitive to Nampt inhibition (Figure 3.1E), but resists expansion upon Nampt overexpression (Figure 2.2D). This relationship may reflect regulated import of NAD into the matrix, about which little is known, perhaps in order to preserve the intramitochondrial redox ratio or satisfy the needs of the TCA cycle. Finally, the apparent rescue of ATP generation following NR administration to mNKO mice (Figure 3.7) indicates that Nampt activity is critically tied to NAD co-factor-dependent steps in glycolysis and oxidative phosphorylation and can be functionally replaced by an alternate source of NMN. Accordingly, this work generally classifies Nampt as a critical buttress of oxidative metabolism in muscle, rather than a master activator.

*Implications of Enhanced Nampt Function in Muscle*

The idea of targeting NAD homeostasis as a potential regulator of the expression, stability, or chromatin binding of PGC-1a in muscle emerged when it was discovered that the co-activator is a target of Sirt1 in this tissue (Gerhart-Hines et al., 2007). Despite its seemingly
counterintuitive nature, the idea that a single transgene might enhance the oxidative performance of otherwise healthy muscle tissue is not without precedent. In the most famous example, overexpression of PGC-1a (encoded by Ppargc1a) in muscle was sufficient to shift a majority of type II glycolytic fibers to type I oxidative fibers, resulting in fatigue resistance and induction of a transcriptional program responsible for mitochondrial biogenesis (Lin et al., 2002). Muscle-specific overexpression of CamKIV also increased both muscle mitochondrial content and whole body insulin sensitivity by appearing to act, at least in part, by stimulating expression of PGC-1a (Lee et al., 2014; Wu, 2002). However, despite the identification of novel isoforms and splice variants of Ppargc1a, a decade’s worth of attempts to activate the factor by indirect genetic means have largely fallen short (Ruas et al., 2012). Our finding that Nampt overexpression does not increase expression of Ppargc1a and is not sufficient to shift the energy balance or increase the endurance of young mice (Figure 2.2-2.4) can likely be counted among these attempts, simply indicating that NAD synthesis is not the lone factor, among dozens of potential candidates, which limits oxidative capacity in young muscle. Concurrent work from an independent laboratory concluded that muscle-specific expression of Sirt1, a presumed activator of PGC-1a, is similarly insufficient to affect wholebody energy balance (White et al., 2013). Though the popular model of NAD-dependent muscle remodeling is clearly incomplete, corollaries of these conclusions provide additional insight into muscle metabolic homeostasis.

One aspect of muscle metabolism unveiled by the mNTG model is the limited capacity for transcriptional regulation to enlarge the NAD pool. It is accepted that Nampt activity is at least partially stimulated through increased transcription following activation of AMPK during energetic stress (Cantó et al., 2010), and that the first and rate-limiting step of any biosynthetic pathway is an ideal node for flux regulation. However, above a certain threshold, the distinctly non-linear relationship that we detected between muscle Nampt expression and NAD content (Figure 2.1) provides strong in vivo evidence for either the depletion of the PRPP precursor pool, or the presence of a negative feedback mechanism acting on the enzyme itself. Such feedback is completely overlooked by recent literature, yet has been postulated by in vitro asaays of enzyme
kinetics (Burgos and Schramm, 2008). Indeed, the synthesis of other nucleotides, such as purines and pyrimidines, is known to be regulated by small molecule allosteric interactions at several enzymatic steps (Lane and Fan, 2015). By competitively inhibiting Nampt activity, NAD and NADH may effectively limit the energy committed to their own synthesis and ensure that NADH does not become diluted to the point of energetic crisis. Interestingly, physiologists have speculated about the existence of such a mechanism for many years. In fact, in the late 1960s, some of the first studies in rat liver of NMN pyrophosphorylase, the enzyme that would later become known as Nampt, identified end-product inhibition of the enzymatic activity by pyridine nucleotides (Dietrich et al., 1968). Additional connections abound. Contemporaries of these authors had recently identified a similar mechanism in the ten-step histidine biosynthetic pathway, in which the first reaction, catalyzed by another phosphoribosyltransferase, called ATP-PRTase, was potently inhibited by both AMP and histidine (Martin, 1963). After structural studies of at least 17 different phosphoribosyltransferase enzymes were completed, we now know that ATP-PRTase and NamPRTase (another name for Nampt) together constitute half of the “type II” subfamily, defined by atypical features (Sinha and Smith, 2001; Wang et al., 2006). Thus, it is distinctly possible that small molecule allosteric regulators also dictate Nampt activity in vivo, and that our work has uncovered the limits of NAD synthetic regulation by genetic means in muscle. Such intrinsic factors, and even potentially novel PTMs, warrant further investigation, as they may be vital to the action of Nampt during exercise or calorie restricted states.

The most interesting consequences of muscle Nampt overexpression, by far, were not apparent until the mNTG animals had aged for two years. In this context, it is clear that the ability to maintain NAD synthesis at least partially preserved the treadmill performance of mice (Figure 3.9) in a manner strongly reminiscent of the effects of CR in aged animals (Baker et al., 2006; Hepple et al., 2005). Despite the end-product inhibition effect that may create an upper limit on the Nampt-mediated NAD pool, supraphysiological levels of Nampt protein are still able to powerfully defend against insults that might contract the pool. Thus, whether increased consumption or decreased salvage pathway activity drives the age-related decline in
intramuscular NAD, our data from aged transgenic mice confirm the prediction that the pool should be protected. Furthermore, youthful steady state NAD levels in the aged mNTG mice (Figure 3.9) allow us to deduce that the activity of NMNAT1-3, together constituting the second step in the salvage pathway, does not likely become rate-limiting to the pathway over time.

Which NAD-dependent processes, then, are maintained in aged mNTG muscle?

Considering that Ckmm-Cre is expressed at low levels in the heart, one might predict that Nampt promotes exercise performance by slowing an age-related decline in cardiac output. While this decline may be present in humans, the cardiac output of rodents has rather been shown to increase with age, making this mechanism unlikely (Delp et al., 1998). With regard to the redox state, a slight decline in pyruvate content (Table 3.1) may indicate that aged mNTG muscle maintains slightly more reduced cytosol. Skeletal muscle of mNTG mice may also benefit from preserved antioxidant defenses, which fit into the free radical theory of aging by sparing oxidative damage to DNA or sensitive aspects of the contractile apparatus, such as the ryanodine receptor (Umanskaya et al., 2014). In the simplest such mechanism, maintaining the pool of NADPH might allow reduced glutathione to drive the detoxification of hydrogen peroxide (Lass et al., 1998).

Post-translational mechanisms might also involve decreased ROS production or the activation of superoxide dismutase via mitochondrial Sirt3 (Jing et al., 2011; Qiu et al., 2010).

Aging also causes a decline in the number of motor neurons in type II fibers, in particular, which may be connected to the observation that ROS production can alter the structure and function of the neuromuscular junctions (Gonzalez-Freire et al., 2014). A significant and growing body of literature suggests that neuronal insults are mitigated by local NAD synthesis (Araki et al., 2004; Fang et al., 2014; Scheibye-Knudsen et al., 2014; Wang et al., 2014), some of which may diffuse from mNTG myofibers into surrounding nerve cells. Interestingly, neurological aspects of premature aging that accompany PARP hyper-activation in Cockayne Syndrome can be mitigated by NR or BHB, strongly implicating energetic stress secondary to NAD and ATP depletion as causal factors in this disease (Scheibye-Knudsen et al., 2014). Since almost all organs are
innervated to some degree, the central nervous system is a prime target of NAD-boosting treatments that may explain many of the effects of systemic precursors.

Finally, muscle transcriptional programs relating to mitochondrial biogenesis or substrate selection are popular candidates for combating aging. However, our data indicate that only 18 genes are differentially expressed between 24-month-old mNTG and the wildtype, strongly suggesting that the phenotype is driven more by metabolic flux than by a set genetic program. The same data do provide tantalizing evidence that 500 genes change over the course of aging in a manner than is unique to either control or mNTG mice (Figure 3.9H), raising the possibility that the transgene is responsible for altered transcription. However, the genes on this list may serve only as fodder for speculation, as it is difficult to make a statistical argument that NAD homeostasis renders a given gene significantly unchanged over time in one group or another. Indeed, the relationship between muscle NAD and aging may be tested more easily by asking whether phenotypes that resemble sarcopenia can be achieved on a shorter time scale by specifically inhibiting Nampt.

*Implications of Diminished Nampt Function in Muscle and NAD Precursor Treatment*

The mNKO mice represent a unique, titratable model of metabolic stress that will serve as a valuable reagent to muscle physiologists. The energetic crisis experienced by mNKO muscle fibers nearing 7 months of age is likely a large-scale manifestation of what was first described as “PARP-mediated cell death” as early as 1983, following exposure of cells to genotoxic agents (Sims et al., 1983). It was not until the generation of a floxed Nampt allele in 2008 that it became technically feasible to lower NAD in selected tissues without first hyperactivating PARPs by damaging DNA (Rongvaux et al., 2008). Such a system allows investigators to isolate the effects of NAD deficiency by limiting production of the dinucleotide.
Using the floxed allele, our mNKO model revealed several interesting aspects of NAD dynamics in muscle. First, as bulk NAD declines in muscle, cytosolic NAD drops below the $K_m$ for GAPDH to inhibit glycolysis in at least a subset of fibers. Below this threshold of approximately 60 µM NAD (Lambeir et al., 1991), more carbon is shunted into the pentose phosphate pathway and accumulates most significantly in the form of seduheptulose 7-phosphate, forming a potentially useful biomarker of NAD restriction. Second, the mitochondrial NAD pool tends to reflect that of the cytosol and is not protected in the manner observed in cell culture studies using genotoxic agents or the Nampt inhibitor, FK866 (Pittelli et al., 2010; Yang et al., 2007a). This suggests that enzymes of the TCA cycle and mitochondrial sirtuins are susceptible to restricted activity in states of declining NAD. Of course, without sufficient ATP production by OXPHOS, subtle variations in the signaling activity of sirtuins and PARPs are likely moot. Third, the hyperbolic relationship between mitochondrial NAD and OXPHOS activity indicates the existence of a narrow NAD threshold, above which the electron transport chain appears to function optimally (Figure 3.7D). Our approximations in isolated muscle mitochondria place the theoretical maximum oxygen consumption at 200 pmol/sec/mg mitochondrial protein and the half-maximal NAD concentration at 0.4 nmol/mg. This NAD threshold is also below the best published estimate of steady state intramitochondrial NAD, measured at 2.0 nmol/mg mitochondrial protein using mass spectroscopy in HEK293 cells (Yang et al., 2007a), and is likely set by the TCA cycle dehydrogenase with the highest $K_m$ for NAD. Fourth, below the critical threshold, the mechanism of cell death is unregulated. This tissue-based observation is highly consistent with the behavior of cells under genotoxic stress, since the energetic demands of apoptosis render necrosis a more common route of PARP-mediated cell death (Nicotera et al., 1998). The activation of muscle-specific ubiquitin ligases is a nearly uniform feature of muscle wasting disorders that is absent in mNKO muscle, providing an unusually strong indication of proteosome-independent catabolic processes at work. Finally, and most importantly, nicotinamide riboside (NR) administration is capable of functionally restoring NAD-depleted muscle (Figure 3.7, 3.8). This critical finding suggests that NAD precursors in the circulation are capable of being taken up and utilized by
muscle to support ATP generation. The dynamics of this process, however, are surprisingly complex.

Given the profound effects of NR treatment in mNKO mice, one might anticipate that muscle NAD content would be completely restored by this treatment. However, our observation that NAD is only fractionally restored by NR, despite a complete rescue of ATP production (Figure 3.7, 3.8), indicates a puzzling non-linear relationship. Importantly, a similar effect appears in at least three other studies, in which myopathy models that do not exhibit baseline NAD depletion are, nonetheless, rescued by dietary NR (Cerutti et al., 2014b; Khan et al., 2014b; Xu et al., 2015).

The seemingly paradoxical behavior of NR in vivo is likely driven by a combination of three factors. First, even the relatively high NR dose of 400 mg/kg/day is poorly bioavailable to muscle. Our orally-administered isotopomer studies indicate that intact NR does not, in fact, reach the muscle within 100 minutes, though a portion of the intramuscular NMN pool and a majority of the NAM pool are NR-derived by this time (Figure 3.7). Furthermore, only a trace amount of the [M+2] NAD isotopomer was detectable in a single sample of mNKO muscle following administration of labeled NR, indicating that NR may have remarkable little direct influence on this pool, contrary to the prevailing model (Cantó et al., 2012). A second consideration is that bulk tissue NAD measurements may be too coarse to detect the specific local elevations responsible for the effect. A likely example is the mitochondrial matrix, which cumulatively represents only about 10% of the tissue volume, but maintains an NAD concentration as much as twenty-fold higher than the cytosol (White and Schenk, 2012). Given that mitochondria are crucial to NAD-dependent ATP synthesis, it is conceivable that they actively transport the dinucleotide to receive preferential access (Yang et al., 2007a). Accordingly, under the conditions that we tested in NR treated mNKO mice, the approximate doubling of bulk tissue NAD (Figure 3.7G) might conceal a larger change in that of matrix itself. Finally, it is important to consider that single measurements of metabolites, even those assumed to be in steady state, do not reflect the dynamic turnover of metabolite pools. In the absence of technical means to
measure flux through the NAD salvage pathway, we must accept the possibility that NR uptake, conversion to NAD, utilization in redox reactions, and degradation to NAM are occurring at the same rate in mNKO muscle as the salvage pathway functions in wildtype muscle. Thus, whole tissue NAD levels need not be restored for NAD-dependent enzymes to function normally in support of tissue homeostasis. The flux of NAM equivalents through a cell could also conceivably contribute to NAD-independent effects, as formation and secretion of methyl-NAM might deplete S-adenyl methionine, the same methyl donor utilized to modify chromatin (Fan et al., 2015). Such explanations offer key insights into the work of other authors, as the mNKO model provides the highest resolution yet for examining the influence of metabolite precursors on the muscle NAD pool.

Can NAD Deficiency Model Progeria?

Part of rationale for producing the mNKO line of mice was to examine whether the resulting phenotype might resemble natural aging. However, even in the most extreme report, hindlimb muscle NAD declines by 40-60% in C57BL/6 mice between six and 22 months of age (Gomes et al., 2013), while we observed a decline closer to 30% over a similar time frame (Figure 3.9). It is not surprising, then, that the massive NAD decline in mNKO mice drives a phenotype more severe than sarcopenia alone. In some regards, the mNKO mice do have progeroid features; muscular stiffness, weakness, decreased fiber diameter, increased fatigability, and bone remodeling are undoubtedly commonly in the elderly (Mitchell et al., 2012; Weinstein and Hutson, 1987). The dramatic upregulation of Cdkn2a is one tantalizing piece of evidence that NAD depletion might drive senescence, though it is possible that this transcript arose in muscle satellite cells, perhaps approaching the limit of their replicative capacity after months of continuously repairing damaged muscle (Sacco et al., 2010). Through an analogous mechanism, another group has utilized the floxed Nampt allele to induce aging-like functional defects in adult
neural progenitor cells (Stein and Imai, 2014a). Yet, the long-standing challenge of isolating the effects of transcriptional regulation from metabolic flux persists in each of these settings.

In our highest resolution analysis, mRNA-seq identified only 41 genes whose transcription changed by more than 1.5-fold in the same direction when comparing twenty months of natural aging to Nampt deletion in young adult mice (Figure 3.5F). While this list is too short to conclude that NAD-deficiency promotes muscle aging through transcriptional regulation, it may provide hints as to the biological processes influenced by NAD, especially considering that almost half of the candidates are protected from age-related change in mNTG muscle. Among these, *Cacng7* and *Cacna2d4* encode subunits of voltage-dependent calcium channels, while *Anxa4* and *Cpne2* encode calcium-dependent phospholipid binding proteins. It is interesting that calcium-sensitive intracellular elements appear on this list, since calcium is also the NAD-sensitive factor most likely to impact muscle function through non-transcriptional mechanisms. Calcium is essential to the excitation-contraction coupling of muscle and may be highly sensitive to NAD homeostasis, though its dynamics in intact tissue are technically challenging to measure. In addition to the potent calcium-mobilizing action of NAD relatives, cADPR and NAADP (Pollak et al., 2007), evidence that pyridine nucleotides interact with voltage gated ion channels (Kilfoil et al., 2013) and ryanodine receptors (Sitsapesan and Williams, 1995) could easily contribute to the characteristic muscle weakness and swollen sarcoplasmic reticula (Figure 3.3) of the mNKO line. This is especially true when considering that calcium is an obligate co-factor for activators of PGC-1α, such as CaMKIV, as well as the dehydrogenases of the TCA cycle, and that the constitutive transfer of this ion from the endoplasmic reticulum to the mitochondrial matrix may be required to maintain mitochondrial energetics (Cárdenas et al., 2010). In an ironic twist, the unique 2-O-acetyl-ADPR metabolite generated by sirtuin activity has also been reported to directly potentiate the calcium-permeable TRPM2 ion channel, providing a means of impacting muscle physiology completely independent of epigenetics (Grubisha et al., 2006). Thus, while not directly addressed in this work, the relationship between NAD metabolism and calcium homeostasis in aging tissues will be an interesting subject of future studies.
The progressively severe phenotype of the mNKO mice limits their utility as a true model of progeria. Indeed, despite poor overlap with aged mice, nearly 90% of the genes differentially expressed in mNKO muscle are also dysregulated in the *mdx* model of Duchene’s Muscular Dystrophy (DMD) (Haslett et al., 2005). This may be the case for many severe muscle injury models, since post-mitotic fibers have a limited repertoire of responses to physical and metabolic insults and are increasingly subjected to immune cell infiltration. Yet, it is interesting to consider whether NAD deficiency might be a primary cause of some dystrophies. It is conceivable that cytoskeletal derangements that increase the fragility of fibers might result in the leak of small molecule metabolites. To date, the only studies to directly examine the NAD content and mitochondrial function of *mdx* muscle independently concluded that NAD levels were significantly lower and complex I insufficiency was responsible for limiting ATP production (Rybalka et al., 2014). Our work indicates that a causal relationship between these observations is highly likely, and may have gone unnoticed for years due to the non-obvious connection between dystrophin morphology and metabolic flux. Furthermore, if particular cytoskeletal interactions are strengthened by NAD-dependent PTMs, such relationships would only become apparent when the ubiquitous metabolite becomes massively depleted, as in the case of mNKO mice. To date, the only such example demonstrated to affect a model organism is ADP-ribosylation of the extracellular region of integrin alpha-7, which appeared to modulate affinity for the laminin ligand when exposed to micromolar concentrations of NAD (Goody et al., 2010; Zhao et al., 2005). Thus, the primary source of muscle wasting in mNKO mice might not be metabolic or transcriptional at all, but more similar to the initiating events of classical laminopathies. It will be an important subject of future work to cross the mNTG line onto the *mdx* background and determine whether the resulting male offspring have a less severe phenotype than mice without the transgene. Such a finding could be a groundbreaking advance for the treatment of DMD through NAD-based therapeutics.

Finally, three decades of literature regarding NAD depletion have dealt overwhelmingly with the cellular response to genotoxic stress (Kraus, 2015). Though potentially applicable to
aging, our finding that the mNKO phenotype is reversible may be most relevant to the treatment of natural disease states in which muscle responds to massive DNA damage by hyperactivating PARPs. Rare mitochondrial myopathies that induce ROS formation (Wallace and Fan, 2009) might be good candidates. However, a more frequent example of DNA damage in muscle is following cancer chemotherapy. Anthracyclins, the commonly prescribed class of anti-tumor drug, which includes doxorubicin, has a high affinity for inner mitochondrial membranes and is known to cause long-lasting cardiotoxicity after being sequestered in muscle (Guaspillou et al., 2015). Interestingly, this cardiotoxicity can be abrogated by deletion of PARP1 or co-administration of pharmacological PARP inhibitors (Pacher, 2002), suggesting that NAD-depletion is a component of the mechanism. If similar mechanisms are responsible for the dose-dependent decrease in muscle twitch force following doxorubicin treatment (Hydock et al., 2011), NAD precursors might help alleviate the associated fatigue in patients. In this case, the addressable population for NAD-based therapeutics might be expanded to those being treated for hematological malignancies and solid tumors.

**Significance and Conclusions**

Our work also sheds light on the complex and dynamic nature of NAD metabolism in muscle and provides new tools that may now be used to dissect old problems. For example, we initially set out to challenge the assumption that CR shifts the NAD/NADH redox state of muscle, in part, by boosting Nampt expression. By applying modern mass spectroscopic techniques to the classical metabolite indicator method (MIM) of redox analysis, we demonstrated that Nampt is not sufficient to induce such a change (Figure 2.5). Our finding calls into question whether the only report to identify a CR-induced redox shift in muscle (Chen et al., 2008) should be revisited using the MIM, as others have also noted inconsistencies between this model and classic literature (Kaelin and McKnight, 2013).
From a therapeutic standpoint, the ability of NR to stimulate muscle remodeling is a particularly promising finding, given that naturally occurring compounds have the potential to be rapidly developed for use in patients as nutriceuticals. However, several key questions must first be answered. It is clear that NR exerts a powerful influence on the physiology of NAD-depleted muscle over the 6-week timeframe that we tested; yet, the durability and associated toxicity of chronic treatments remains unknown. As preliminary safety trials in humans are currently being conducted, an oral NR dose of up to 3000 mg/kg/day was recently shown to have a similar toxicity profile to an equimolar dose of nicotinamide, when administered to rats for up to 90 days (Conze et al., 2016). While these data are promising, a similar safety profile would likely need to be maintained for months or years in order for NR to be applicable to the treatment of chronic diseases. And, while mixing vitamins into food or drinking water is technically simple, alternative routes of administration might alter the way the way NR is ultimately utilized. For example, intravenous administration may allow the molecules to effectively bypass the flora of the digestive tract, as well as the detoxification mechanisms of the liver, to exert more powerful influence on periferal tissues. Further investigation into the physiological properties of this compound is surely justified.

With regard to the future of NAD-mediating therapeutics, the efficacy of NR may serve as a starting point for further technical innovations. The ability of NR to rescue metabolic function in the mNKO mice without restoring wildtype bulk NAD levels may inform the development of novel NAD-mediating strategies by altering the desired endpoint. In particular, the pursuit of acid, amide, and ester derivatives of NR may have been hampered by initial findings that these compounds elevated the NAD of cultured cells to a lesser extent than the parental molecule (Yang et al., 2007b). Our work gives reason for these and other NR derivatives to be revisited as potential therapeutics, especially if they exhibit improved stability or pharmacological properties.

A final remaining technical hurdle is the assessment of the kinetics of NAD turnover to NAM and back, which is highly relevant to NAD-dependent physiology, but has never been measured in tissues. Using the [M+2] isotopomer of NR designed and synthesized by our
collaborators, it is now possible to deduce how recently a given molecule of NAD was produced by the salvage pathway, allowing us to measure the rate of NAD turnover in C2C12 cells in preliminary tests. After further optimization, including a way to steadily infuse the labeled NAD precursor and repeatedly sample from the same tissue, this approach might be applied to measurement of actual NAD turnover in vivo. Such a technique might finally identify the source of age-related loss of NAD in multiple tissues and determine whether CR blunts this effect.

These studies are the first to experimentally demonstrate an essential role for muscle NAD metabolism in promoting mammalian health. With regards to combating obesity or enhancing the performance of otherwise healthy muscle, stimulation of the existing NAD salvage pathway in muscle does not hold promise. This approach is not without merit in other tissues, however, as preliminary work from our lab suggests that Nampt overexpression in the hypothalamus may limit diet-induced weight gain. And, since the recent discovery of P7C3, a small molecule that was demonstrated to bind and activate Nampt (Wang et al., 2014), mechanistic and tissue-specific effects of Nampt activation are the subject of increasing attention. Excitingly, Calico, a well-funded biotechnology company, is currently exploring the neuroprotective properties of P7C3 for clinical applications.

Our examination of the aged mNTG mice presents the equally exciting prospect that age-related loss of NAD homeostasis is a preventable cause of tissue dysfunction. In light of the dramatic impact of NAD precursors that we have demonstrated in muscle, the stage is set to test the ability of chronic NR administration to preserve the function of this, and perhaps other tissues, into old age. Finally, NAD boosting therapeutics may one day dramatically improve the lives of people suffering from congenital muscular dystrophies or chemotherapy-induced muscle weakness. The central role of NAD metabolism in skeletal muscle has long been appreciated, yet our understanding of the ways in which it may be manipulated to promote human health is only in its infancy.
Figure 4.1. Comprehensive working model relating muscle function to muscle NAD content. The age-related decline in muscle NAD is preventable with sufficient dosage of the Nampt transgene. However, direct NAD-related processes account for only a portion of the functional decline. Over a shorter time frame, deletion of the Nampt gene from skeletal muscle of young mice appears solely responsible for a dramatic functional decline that can be reversed by administration of NR. Despite exerting minimal influence on the bulk intramuscular NAD pool, NR may specifically restore the NAD-dependent processes of aerobic metabolism in the mitochondrial matrix. Arrows indicate the metrics of young wildtype mice.


Stein, L.R., and Imai, S.-I. (2014b). Specific ablation of Nampt in adult neural stem cells recapitulates their functional defects during aging. EMBO J.


