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Adipose tissue FABP deficiency promotes metabolic reprogramming and positively impacts healthspan

A dissertation presented

by

Khanichi Nona Charles

to

The Committee on Biological Sciences in Public Health

in partial fulfillment of the requirements

for the degree of

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in the subject of

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Dissertation Advisor: Professor Gökhan Hotamişligil MD, Ph.D. Khanichi Nona Charles

ADIPOSE TISSUE FABP DEFICIENCY PROMOTES METABOLIC REPROGRAMMING AND POSITIVELY IMPACTS HEALTHSPAN

ABSTRACT

The adipose tissue lipid chaperones aP2 and mal1, also known as fatty acid binding proteins (FABPs), are significant molecules contributing to metabolic homeostasis, whereby their absence promotes physiological changes that improve systemic metabolism. Identification of palmitoleate as a lipokine generated in aP2-mal1 deficiency—originating from adipose and directing the lipogenic program in liver, established a role for these chaperones in linking adipocyte and hepatic function. We have recently demonstrated a functional role for secreted aP2 in the activation of gluconeogenesis and hepatic glucose output, further designating this molecule as an adipocyte-derived regulatory factor that influences liver metabolism. Key molecules linking the metabolism of nutrients in energy generating pathways are the nucleotide cofactors NAD and NADH. Together, these molecules function to coordinate the maintenance of redox reactions during normal cellular metabolism and act as required substrates for enzymes such as sirtuins and poly ADP-ribose polymerases. Using global metabolite profiling, we show that combined deficiency of the adipose tissue lipid chaperones aP2 and mal1 leads to a hepatic nucleotide imbalance resulting from metabolic

reprogramming in liver. We demonstrate that this reprogramming of metabolite flux is accompanied by significant alterations in liver NAD metabolism and establish a role for aP2 in directing substrate utilization through inhibition of the rate-limiting enzyme for NAD synthesis, nicotinamide phosphoribosyltransferase. Several models for the proposed regulatory pathways that link nutrient metabolism to aging include mechanisms that are NAD dependent. Accordingly, we found that long-term FABP deficiency confers a strong resistance to aging related metabolic deterioration. Together, the findings presented in this thesis support a considerable role for FABPs in the regulation of NAD metabolism and healthspan.

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DEDICATION

For my family: a curious bunch.

The Days, Tapés, Jacksons and my MLC's

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LoLo, Sister Sarah and Micheley and James, our bond endured distance and time. Thanks for the foolishness. And lastly, to my lovely Golden State… thank you for taking me back.

CHAPTER 1

INTRODUCTION

METABOLISM AND AGING

Growing old: healthspan vs. lifespan

From the moment of creation and until death, all forms of life begin to experience the processes of aging. For some species, like the Californian Bristlecone Pine, these processes can occur over thousands of years. For humans and other higher order organisms, the chronology of aging is much shorter, marked by periods of physical and cognitive growth, occurring at time-points that follow the species norm. In advanced adulthood, we begin to experience a decline in these physical and cognitive abilities, as our adaptive responses begin to loose efficacy and we become more susceptible to disease. For these reasons, our perception of age has progressed beyond one that is defined solely by time of existence, towards one that includes the condition of health. Undoubtedly, illness prevention has dramatically increased the average healthspan, however, our maximum lifespan remains limited to roughly 120 years (Ruiz-Torres and Beier, 2005). Though there are many examples of maximal lifespan extension in yeast, *Drosophila* and mice, whether significant human lifespan extension can be achieved beyond the current limit remains to be answered. In fact, dietary restriction, an intervention shown to have lifespan extending effects in yeast, *Drosophila,* and mice, was demonstrated to have limited effects on lifespan in primates (Mattison et al., 2012). Nevertheless, we endeavor to understand what occurs over time, with respect to biological function, to ensure the inevitability of

death. In doing so, we may be able to design interventions that add meaningful years to human healthspan.

Metabolic and molecular links to aging

The impact of reliable food sources, protection from environmental insults, medical advancement, and disease prevention has manifested as a three-fold increase in average human lifespan (National Center for Health Statistics, 2010). However, in today's world, everyday life often includes daily routines marked by inactivity and nutritionally poor meals that are high in calories. Together, these lifestyle adjustments have led to a rise in obesity, negatively impacting healthspan and introducing metabolic syndrome (i.e., insulin resistance, type-2 diabetes, and atherosclerosis) as a new cause of disease related premature death (Katzmarzyk et al., 2005). Though it has been possible to identify behaviors and exposures that lead to metabolic disease, it is difficult to elucidate the underlying mechanisms. This is due to the immense heterogeneity in genetic composition and environmental exposures that contribute to the development of metabolic derangements. Despite this complexity, determining mechanism becomes a more tractable concept when assayed using genetically identical laboratory species. Thus far, such studies have revealed insulin and insulin like growth factor 1 pathway modulation, mammalian target of rapamycin inhibition, and sirtuin

activation as putative interventions that oppose the decline in metabolic function and increase longevity (Cantó and Auwerx, 2011).

The yeast sirtuin, Sir2p was first identified as a mediator of lifespan whereby mutations that inactivate Sir2p decreased survival, and introduction of a second copy of Sir2p conferred a 30% increase in replicative life span (Kaeberlein et al., 1999). It was later determined that expression of Sir2p could be induced by shifting glucose availability from 2% to 0.5%, a method of dietary restriction in yeast. It is hypothesized that this form of DR increases Sir2p by initiating a metabolic switch from fermentation to respiration, creating more NAD from NADH which activates Sir2p, an NAD-dependent deacetylase (Imai et al., 2000; Lin et al., 2000).

Though mammals possess a Sir2p orthologue (Sirtuin 1), the mechanism(s) of increased lifespan in mammals is complicated by the nature of an animal's ability to respond to changes in energy availability through adaptive changes in glucose and fat utilization by metabolic tissues. This adaptive response involves suppression of glycolysis, enhanced liver gluconeogenesis and glucagon-stimulated degradation of glycogen stores in order to maintain glucose levels during short-term caloric restriction. Insufficient availability of carbohydrates for prolonged periods of dietary restriction will stimulate lipolysis and mobilization of lipids from adipose tissue (Dhahbi et al., 2001). Thus, long-term dietary restriction results in lower steady state levels of glucose and insulin as well as an overall decrease in fat mass (Bertrand et al., 1980; Gabriely and Barzilai, 2001).

Dietary restriction in mammals is typically defined as a 25-60% reduction in total calories derived from carbohydrates, lipids, and proteins without invoking a state of malnutrition (Ricketts et al., 1985; Weindruch et al., 1986). Under this intervention, studies have shown life span extension to increase by as much as 50% in dietary restricted rodents (Sohal and Weindruch, 1996) and to date, this phenomenon has held true across numerous species including yeast, spiders, flies, worms, and fish (Ingram et al., 1990). The emerging facts indicate that the benefits of longevity result from a delayed development of age related diseases, many of which have known causal links to metabolism such as type 2 diabetes, cancer and cardiovascular disease (Hursting et al., 2001; Lane et al., 1999; Manco and Mingrone, 2005). However, two dietary restriction studies spanning several decades revealed that *rhesus* monkeys subjected to this intervention displayed great extension of healthspan, but no increase in survival (Colman et. al. 2009 and Mattison et al., 2012). Despite much attention surrounding the life extending effects of DR, the regulatory factors mediating the relationship between a restrictive diet and postponement of mortality is currently unknown.

Genetic models of extended lifespan

Several genetic models for significantly increased lifespan have been reported over the years. Of note are mouse models harboring mutations in growth hormone

receptor ($Ghrhr^{lit/lit}$) or growth hormone receptor binding protein ($GHR/BP^{-/-}$), p66shc null mice, and IGF-I receptor heterozygous mice, all of which exhibit altered hormone secretion in the hypothalamic-pituitary axis (Bartke et al., 1998), and have been shown to affect many of the same physiological changes that occur during aging (reviewed in Liang et al., 2003). Dwarf mice, perhaps the most extensively characterized genetic models of longevity, consist of a collection of mutants that include both Snell and Ames mice, harboring point mutations in the pituitary specific transcription factor 1 gene (Pit1) and the Prop-1 gene upstream of Pit1, respectively. Each of these mutations result in poor secretion of growth hormone, thyroid-stimulating hormone, and prolactin, an outcome related to defective differentiation of their respective hormone secreting cells in the anterior pituitary. In addition to the expected growth phenotype as a consequence of lacking growth hormone, these mice also have a reduced metabolic rate and core body temperature, lower circulating levels of insulin, IGF-1 and glucose, and an overall increase in resistance to oxidative stress (Dominici et al., 2002). Expression profiling of liver tissue in Ames mice, which live up to 40-70% longer than their wild type counterparts (Brown-Borg et al., 1996) and dietary-restricted mice, revealed both separate and overlapping gene clusters that may represent clues towards the molecular basis underlying lifespan extension (Tsuchiya et al., 2004).

Adipose tissue in healthspan and lifespan

Given the central role of adipose tissue in communicating the nutritional environment to peripheral tissues and in directing systemic energy flow, it is unsurprising that major energy shifts, such as one induced by dietary restriction, highly impact adipose tissue function. The opposite is also true, as dramatic alterations in adipose tissue function greatly influence systemic metabolic responses to nutritional inputs. Decreased adiposity is associated with the activation of genes involved in carbohydrate, amino acid, lipid, and mitochondrial energy metabolism; each serving to expend energy. Adiponectin expression, which is reduced in obese humans and insulin resistant rodents (Hu et al., 1996), increases upon loss of fat mass **(**Yang et al., 2001). Furthermore, surgical excision of white adipose tissue reverses hepatic insulin resistance that is induced by obesity and aging (Barzalai 1999 and Gabriely 2002).

Taken together, a reduction in adiposity is followed by increased expression of genes involved in peripheral tissue insulin sensitization and systemic energy expenditure. Yet, many ambiguities remain in determining the relationship between adipose tissue and the aging process. In fat-specific insulin receptor deficient mice, which have ~50% reduction white adipose tissue, similar caloric intake, and significantly longer lifespans than their wild type counterparts (Bluher et al., 2003), lifespan extension mediated by dietary restriction was shown to be inversely correlated with degree of fat loss.

PHYSIOLOGICAL ROLES OF THE ADIPOCYTE IN METABOLIC STABILITY AND PLASTICITY

Conventional Functions

Adipose tissue stores energy in the form of triglyceride (TG) filled lipid droplets, adjusting release of its contents based on the substrate requirements of peripheral tissues. Triglycerides are presented to the adipocyte in the form of dietary chylomicrons or liver derived lipoprotein particles and hydrolyzed through the action of lipoprotein lipase at the cell surface. When peripheral tissue substrate demands trigger hormonal signals that lead to adrenergic pathway activation, free fatty acids are liberated from lipid droplets *via* hydrolysis of TGs. In addition to the adipocyte, matrix fibroblasts, vascular endothelium, and a variety of immune cells all comprise the adipose tissue organ. Together, these cell types actively secrete an array lipids and proteins, termed adipokines, that function as key effectors of adaptive responses to metabolic alterations in peripheral tissues (Figure 1-1). For example, the earliest identified adipokines, leptin and adiponectin, influence energy homeostasis at multiple sites in the periphery. Leptin does so by binding to receptors in the hypothalamus to signal satiety and reduce food intake. Adiponectin receptors are more ubiquitously expressed, and their activation is associated with improved insulin sensitivity and increased fatty acid oxidation. In plasma, high leptin and low adiponectin are strongly correlated with degree of

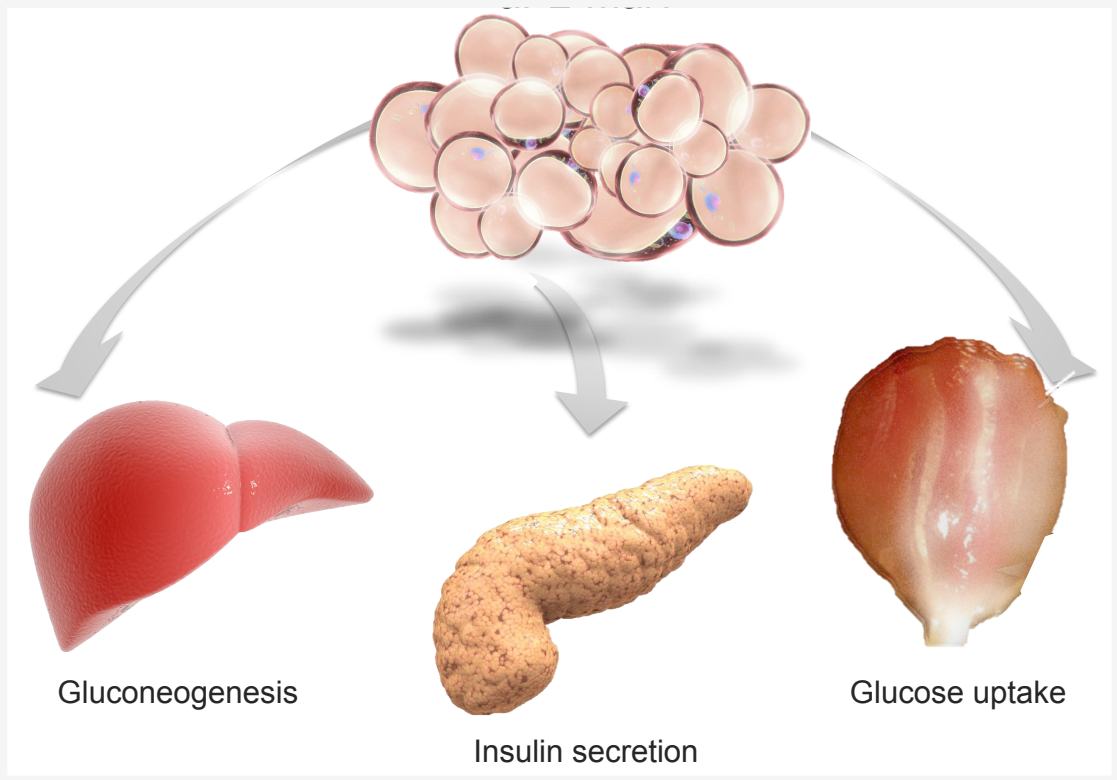

Figure 1-1. Adipose tissue influence on systemic metabolism.

adiposity and insulin resistance. This natural ebb and flow of adipokine secretion and systemic lipid flux occurs most prominently during feeding, fasting and exercise, necessitating a range of adipose tissue adaptive responses to maintain functional integrity of the adipocyte and proper communication with peripheral tissues.

Adaptive responses

The regulation of adipose tissue lipid content during fasting and increased energy expenditure is crucial for supporting the heightened demand for fuel substrates. For example, decreased insulin and increased glucagon stimulates adipose tissue lipolysis and subsequent release of free fatty acids to supply peripheral tissues, as an adaptive response to insufficient food intake. During exercise, the brain signals release of catecholamines, which stimulates lipolytic activity in the adipocyte and supplies working muscle with sufficient substrate levels needed to sustain the high levels of oxidation required for ATP generation. Interestingly, in lactating mice, strong evidence exists that the unfolded protein response transcription factor XBP-1 is required to maintain lipogenic gene expression and milk production by adipose derived mammary tissue, in response to prolactin (Gregor et al., 2013).

The ability to properly access these adaptive responses is affected by a variety of factors; among them are body fat percentage and composition of fatty acids in triglyceride reserves. (Sial et al., 1996, Fabbrini et al., 2012).

Maladaptive responses

Though adipose tissue is capable of expanding to accommodate excess energy, like all tissues, there is a limit to the functional capacity of this organ. Nutritional overload directly impacts adiposity, and induces both qualitative and quantitative changes in the adipocyte lipid droplet. Together these effects trigger anomalous metabolic behaviors, with major consequences on glucose and lipid homeostasis. First, the constant burden of overnutrition overwhelms the capabilities of the adipocyte and leads to ectopic lipid accumulation in circulation and the surrounding periphery. The presence of lipids in tissues that are not functionally equipped to deal with them can trigger dysfunction by exhausting the oxidative capacity of the organ, activating inflammatory mediators, altering organelle membrane composition and depositing toxic lipid species within cellular matrices (Navina et al, 2011). Secondly, endocrine function of many classical secretory organs is compromised in obesity (i.e. pancreatic insulin secretion), including adipose tissue adipokine release (Guilherme et al., 2004). These factors designate adipose tissue as a significant contributor to metabolic disease and, therefore, a

suitable site for identification of molecules that support the maintenance of systemic metabolism.

ADIPOSE TISSUE FATTY ACID BINDING PROTEIN 4

Expression and tissue distribution

The fatty acid binding proteins (FABPs) are a small 14-15kDa family, comprised of 10 members, with ubiquitous tissue distribution. While the degree of functional redundancy for all isoforms remains to be determined, it is known that some tissues strongly express one or multiple isoforms. For example, a single isoform, FABP5 can be found in the eye, whereas the ileum expresses high levels of FABPs 1,2 and 6. In other tissues like testis and adipose, expression is dominated by a single isoform, but also exhibits minor expression of one or more additional FABP family members (Yamamoto et al., 2009). The predominant adipose tissue isoform is FABP4 or adipocyte protein 2 (aP2). Expression of aP2 correlates with fat accretion, as levels rise during adipocyte differentiation as well as in the inflammatory and obese states (Hotamisligil, 2006). Also expressed in adipose tissue is FABP5, or epidermal fatty acid binding protein (mal1). Each of these isoforms are co-expressed in adipocytes and macrophages, however adipocyte aP2 expression is roughly 10, 000-fold higher than macrophage aP2 expression (Shum et al., 2006).

Lipid chaperoning activity

Adipocyte fatty acid binding protein, aP2, preferentially binds long chain fatty acids, and has a weaker affinity towards shorter chain fatty acids and some carboxylate derivatives (Furuhashi and Hotamisligil, 2008). The putative role of cytosolic fatty acid binding proteins, based on their ability to bind fatty acids and other lipids species, is to facilitate the intracellular trafficking of lipids to various organelles and cellular structures for their use as fuel substrates, membrane components, precursors for more complex lipids, or for storage in the lipid droplet. They may also function to present lipid ligands to proteins in the nuclear receptor family for downstream transcriptional activation. Adipocyte fatty acid binding protein aP2 has been linked to a variety of biological mechanisms, as mediators of both lipid trafficking and lipid-derived inflammatory signaling. For example, re-localization of aP2 from the cytosol to nucleus has been observed upon exposure to fatty acid ligands known to function as peroxisome proliferator-activator receptor gamma (PPARγ) targets. indicating a potential role for aP2 in transcriptional activation by nuclear receptors (Nguan-Soon Tan et al., 2002). Furthermore, Erbay et al. (2009) demonstrated that macrophage aP2 was an important mediator of lipid-induced ER stress, and that macrophage aP2 deficiency was sufficient to protect against apoptosis and atherosclerosis caused by lipotoxicity.

Impact on systemic metabolism

Mice deficient in adipose tissue fatty acid binding proteins aP2 and mal1 demonstrate a surprising level of protection against obesity, and the development of atherosclerosis, insulin resistance, and type 2 diabetes when given a high-fat diet (Hotamisligil et al., 1996; Maeda et al., 2005). This is not surprising, given that adipose tissue is a crucial metabolic site, essential to the integration of signal inputs generated in the context of multiple dietary paradigms such as high-fat diet, short-term fasting, and long-term caloric restriction. Furthermore, adipose tissue fatty acid binding proteins facilitate the intracellular movement of lipids for their use in membrane formation, metabolic signaling and storage, all of which have the potential to impact the endocrine and systemic lipid trafficking functions of the adipocyte (Furuhashi and Hotamisligil, 2008).

Work from our lab and others has demonstrated a profound impact of FABP deficiency on adipose tissue and plasma lipid composition (Baar, et al., 2004; Maeda et al., 2005; Cao et al., 2008), and a unique ability for FABP deficiency to promote secretion of a specific lipid species, palmitoleate (Cao et al, 2008). The absence of aP2 and mal1 stimulates adipose tissue *de novo* lipogenesis and synthesis of palmitoleate, a monounsaturated fatty acid with minor dietary orgins (Cao et al., 2008). This lipid was demonstrated as a novel adipokine having hormone like effects, as evidenced by its ability to suppress hepatic lipogenesis and increase insulin-mediated glucose uptake in muscle.

While these proteins have been thought to be mainly cytosolic, given that many of the striking phenotypes in adipose tissue FABP deficiency were observed in the periphery, it seemed reasonable that these proteins could also be secreted to function in an endocrine manner. As such, secreted aP2 was recently demonstrated to regulate hepatic gluconeogenesis through upregulation of gluconeogenic gene expression (Cao et al., 2013).

Together, these data exposed two important features of the adipocyte. First, it revealed the capability of adipocytes to emit specific lipid signals that directly impact systemic metabolism. Secondly, it designated FABPs as molecules that link adipose tissue metabolism to peripheral tissue function.

PREVIEW OF THESIS

By developing the adipose tissue organ, we have evolved distinct mechanisms to both amass and re-distribute energy surplus. However, the efficient regulation of adipose tissue lipid content still requires the complex integration of environmental, hormonal, and nutritional inputs from the entire organ system. In the next 10 years, over half of the human population is expected to be obese. Thus, a fundamental understanding of the adipose tissue contribution to systemic metabolism is crucial to our ability to understand the functional limitations of this organ, and prevent the damaging effects of obesity on peripheral tissues.

Our approach is to examine the role of adipose tissue FABPs in the regulation of systemic metabolic circuitry through global metabolic profiling by using a mouse model deficient in the FABPs aP2 and mal1. Furthermore, given that these animals exhibit a remarkable protection against obesity and the associated co-morbidities, we aimed to use this genetic model to aid in our efforts in determining how protection from a decline in metabolic tissue function impacts survival and healthspan.

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CHAPTER 2

ADIPOCYTE LIPID CHAPERONE REGULATES NAD METABOLISM AND DIRECTS SUBSTRATE UTILIZATION IN HEPATOCYTES

BACKGROUND

Adipose tissue fulfills multiple roles in the orchestration of systemic substrate flux. Foremost, it acts as a rheostat for the circulating free fatty acid environment, balancing storage with lipolytic activity according to the needs of the organism. The endocrine function of adipocytes is becoming more widely appreciated, with the finding that they have the ability to secrete an ever-expanding array of factors that influence metabolic programs in peripheral tissues (i.e. adiponectin, leptin, TNFα, Nampt /Visfatin, and palmitoleate) (MacDougald and Burant, 2007; Hotamisligil et al., 1993; Revollo et al., 2007; Cao et al., 2008). Thus, adipocyte function and peripheral tissue regulation are tightly linked. Since these links are deeply rooted in the functioning of biochemical pathways, and in the flux of nutrients on which these pathways rely, excess nutritional substrates present a challenge to the integrity of these metabolic networks.

Unresolvable metabolic stress is a well-described feature of obesity, marked by dysregulation of major pathways used for routine management of nutritional energy. In liver, aberrant glucose and lipid metabolism present in obesity causes hepatic steatosis, increased glucose output, and increased lipoprotein particle formation. Together these contribute to the emergence of several obesity related co-morbidities, namely type-2 diabetes and cardiovascular disease (Malnick and Knobler, 2006), both contributing to and resulting from dysregulated glucose utilization and lipid metabolism in metabolic tissues.

Much work has been done to identify mechanisms that support the maintenance of metabolic networks disrupted in obesity (Khan et al., 2006; Van Gaal et al., 2006). We have chosen to approach this question by examining the role of adipose tissue fatty acid binding proteins (FABPs) in the regulation of metabolic circuitry. The FABPs aP2 and mal1 have the potential to regulate the localization and availability of fatty acids (Furuhashi et al., 2008), and are perhaps required, at least in part, for the incorporation of lipids into the metabolic network. As such, mice with combined genetic ablation of aP2 and mal1 are resistant to the deleterious effects of high fat diet and obesity (Maeda et al., 2005). A collection of studies has elucidated many of the phenotypic alterations that arise under adipose tissue FABP deficiency. Specifically, mice lacking aP2 and mal1 show a marked protection against hepatic lipid accumulation, the development of type-2 diabetes and insulin resistance when placed on a high-fat diet (Maeda et al., 2005) and also exhibit a remarkable resistance to cardiovascular disease (Makowski et al., 2001). This link between FABP deficiency and improved disease outcomes has also been established in humans, where subjects displaying an aP2 promoter region polymorphism resulting in lower adipose tissue aP2 levels present with a decreased risk for hypertriglyceridemia, type 2 diabetes and cardiovascular disease (Tuncman & Erbay et al., 2006). Despite this body of work, it is likely that additional mechanisms related to FABP action remain undetermined, given that these chaperones influence the availability of such a crucial substrate. Indeed, more recently our lab demonstrated that aP2 is a bona fide adipokine that regulates

hepatic glucose output by increasing gluconeogenic gene activity, a phenotype that is consistent with diminished hepatic glucose output in aP2-mal1 deficiency (Cao et al, 2008; Cao et al., 2013). Also prominent in aP2-mal1$^{-/-}$ animals are decreased liver lipogenesis and increased energy expenditure (Maeda et al., 2005).

Key molecules linking the metabolism of nutrients in energy generating pathways are the nucleotide cofactors NAD and NADH. Together, these molecules function to coordinate the maintenance of redox reactions during normal cellular metabolism, and act as required substrates for enzymes such as sirtuins (Sirts) and poly ADP-ribose polymerases (PARPs). Dietary regimens known to have positive effects on age-related health outcomes have been demonstrated to function in part through the modulation of NAD/NADH. Most importantly, these nucleotide cofactors are required for the maintenance of redox reactions in energy generating and energy consuming pathways throughout normal cellular metabolism (i.e., glycolysis, TCA-cycle, fatty acid oxidation, electron transport).

In mammals, the primary site for NAD synthesis is liver; it is produced either de novo from tryptophan or through salvage pathways involving the recycling of nicotinamide, or nicotinic acid. Nicotinamide phosphoribosyltransferase (Nampt) catalyzes the formation of nicotinamide mononucleotide, an immediate precursor to NAD and the rate-limiting step in the synthesis of NAD from nicotinamide (Imai, 2009). Nampt transcription has been shown to follow a circadian rhythm, protein abundance oscillating in a pattern that coincides with feeding and fasting (Ramsey et al., 2009). However, to date, not much is known about the metabolic signals

contributing to Nampt regulation. In liver, glucose and peroxisome proliferator-activated receptor alpha activation both have been demonstrated to suppress Nampt expression through undetermined mechanisms. At the mechanistic level, Nampt was identified as an important mediator of lipid metabolism in liver, and a direct transcriptional target of hepatic FoxOs.

In this study, we explored the metabolic landscape of FABP deficiency and determined the existence of altered substrate utilization in hepatocytes. We provide evidence of metabolic reprogramming in FABP deficiency, marked by decreased glucose utilization and enhanced fatty acid oxidation; the latter supported by an increase in NAD synthesis via upregulation of rate limiting enzyme, nicotinamide phosphoribosyltransferase (Nampt). Furthermore, we demonstrate that aP2 inhibits Nampt expression, and exerts additional influence on NAD/NADH nucleotide balance by increasing glycolytic rate and suppressing fatty acid oxidation.

RESULTS

Robust impact of FABP deficiency on liver metabolome

Given the complexity of potential outcomes resulting from the perturbation of a single node in a metabolic network, we sought to provide a comprehensive profile of metabolites altered in FABP deficiency using metabolomics. For this, we employed targeted liquid-chromatography/mass spectrometry (LC/MS) of nearly 300 metabolite species and analyzed white adipose, plasma, skeletal muscle and

liver from wild type and FABP-deficient mice using the web-based metabolomic platform, MetaboAnalyst (Xia and Wishart 2011). Comparison of normalized peak signals by volcano plot revealed that adipose tissue FABP deficiency evoked only minor changes in adipose, plasma, and skeletal muscle, but in liver more than 40% of metabolites were altered (Figures 2-1A,B,C,D left panel).

In order to ascribe discernable physiological roles to each of the liver derived factors identified by significance testing and illustrated by heatmapping (Figures 2-1A,B,C,D right panel), we utilized the well-annotated metabolite set enrichment capabilities of MetaboAnalyst. We found that the metabolite sets displaying significant enrichment (red bars) were those related to metabolism of protein (i.e., protein biosynthesis, urea cycle, ammonia recycling), citric acid cycle, and nicotinate /nicotinamide metabolism (Figure 2-2A). Pathway impact analysis was employed to identify metabolite sets with members that reside at highly impactful nodes for generation of the relevant product species. Metabolite sets are plotted such that those existing highest and closest to the diagonal contain the most significant and impactful metabolites (Figures 2-2B). Based on this analysis, nicotinate /nicotinamide metabolism is the most significant and highly impacted metabolic pathway in FABP deficiency.

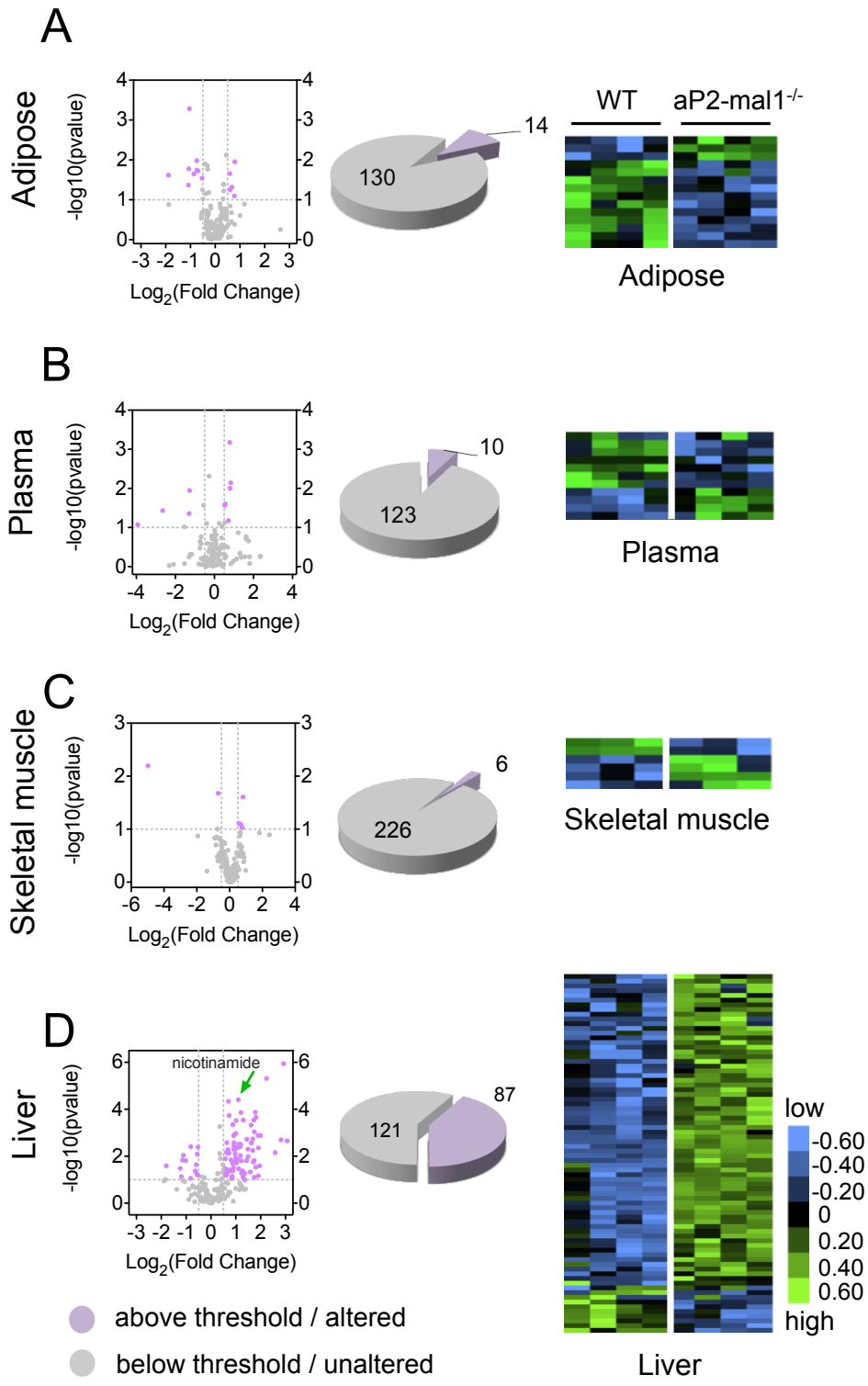

Figure 2-1. Global metabolite profiling.

Figure 2-1 (Continued). Global metabolite profiling.

(A-D) All significantly regulated metabolites are represented in adipose, plasma and skeletal muscle. For liver, only the top 50 metabolites measured are shown. The adjacent pie graphs indicate the percentage of regulated metabolites out of those measured.

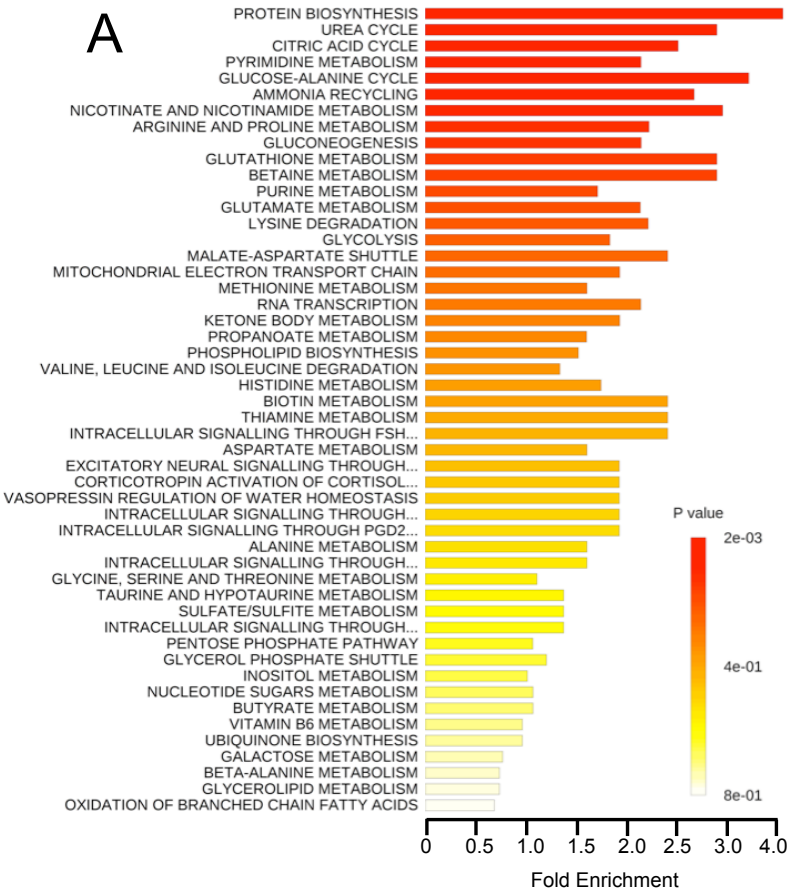

Figure 2-2. Pathway impact analysis of significantly altered metabolites

Figure 2-2 (Continued). Pathway Analysis of significantly altered metabolites

(A) Metabolite enrichment set overview. P value and fold change threshold equal to $p< 0.05$ and 2, respectively. P value corresponds to significance of fold enrichment. Highest significance is indicated in red, lowest is indicated in yellow. (B) Pathway impact. In this graphic, as in 3E, the color signifies pathway significance based on p-value threshold <0.05. Circle size indicates magnitude of pathway impact. 1) Nicotinate /nicotinamide metabolism, 2) Arginine and proline metabolism, 3) Pyrimidine metabolism, 4) Ala, Asp, Glu metabolism, 5) Citric acid cycle.

Liver nicotinamide metabolism and NAD/NADH ratio is altered in FABP deficiency

Nicotinate and nicotinamide are important precursors for the production of NAD, an essential nucleotide and a vital co-factor in the catabolism of lipids, carbohydrates, and proteins. For this reason, we used qPCR to examine hepatic gene expression of enzymes involved in producing NAD, requiring either nicotinate, nicotinamide, or the de novo precursor, tryptophan. Adipose FABP deficiency led to increased expression of de novo NAD synthesis enzymes, tryptophan dioxygenase (1.6-fold), and NAD synthase (2-fold, $p<0.01$) (Figure 2-3A). Contribution of the nicotinate salvage pathway to cellular NAD levels is considered relatively minor, and accordingly we determined no significant regulation of nicotinic acid phosphoribosyltransferase (Naprt), an enzyme specific to this arm (Figure 2-3A). On the other hand, Nampt, a rate-limiting enzyme of the nicotinamide salvage pathway and main route for NAD synthesis in mammals was significantly upregulated (1.8-fold, $p<0.05$). Given that nicotinate /nicotinamide metabolism was identified as the most highly impacted metabolite set, and we did not detect significant gene regulation of the nicotinate pathway, we focused on the NAD salvage pathway requiring Nampt.

Analysis of known transcriptional regulators of Nampt expression revealed significant increases of Foxo1, PGC1α and Bmal in liver of FABP deficient mice. (Liu 2007, Ramsey 2009) (Figure 2-3B). Together, these transcription factors integrate metabolic signals and the molecular clock machinery corresponding to circadian rhythm, in an effort to maintain stability between nutrient availability and cellular

energy demands. In the fed state, Nampt protein levels were increased in aP2-mal1$^{-/-}$ mice compared to wild type (Figure 2-3C), which was concordant with gene expression data (Figure 2-3A). Nampt protein levels highly reflect the cellular energy status, and normally become elevated upon fasting in vivo, or glucose withdrawal in vitro (Imai 2000). However, aP2-mal1$^{-/-}$ mice maintained a constitutively high level of Nampt protein independent of fed status, comparable to wild type levels in the fasted state (Figure 2-3D). Furthermore, the immediate product of Nampt catalytic activity and the precursor to NAD, nicotinamide mononucleotide, was present at high levels compared to wild type in both the fed and fasted state.

Elevated levels of Nampt in aP2-mal1$^{-/-}$ animals is perhaps indicative of an attempt to both restore NAD levels to avoid depletion during excessive consumption and to eliminate excess nicotinamide, an inhibitor of NAD consuming enzymes (Figure 2-3F) (Revollo et al., 2007). To explore this possibility, we first measured nucleotide concentrations using a fluorometric assay to determine the status of oxidized (NAD^+) and reduced (NADH) forms of NAD. FABP deficiency resulted in an overall increase in the total pool of nucleotide (Figure 2-3G). However, NAD^+ content was lowered in aP2-mal1$^{-/-}$ mice (mean difference from wild type, -2.648 ± 0.4 nM/µg protein, $p<0.001$), and was accompanied by a concomitant increase in NADH (mean difference from wild type, 18.10 ± 2 nM/µg protein, $p<0.001$) (Figure 2-3H), corresponding well with the metabolomics data. Interestingly, altered NAD/NADH ratios have been suggested to underlie the

effects of dietary restriction in increasing healthspan (Imai 2009). Specifically, a reduced NAD/NADH ratio largely driven by an increase in NADH levels was reported in livers of dietary-restricted mice (Chen et al., 2008). Here, we observe a similar trend in FABP deficiency, along with other features common to metabolic reprogramming events. With this in mind, we also considered the role of NAD consumption in this process. First, we selected two well-described NAD consuming enzymes to examine: Sirtuin1, and poly-ADP ribose polymerase (PARP). Sirt1 couples NAD cleavage to the deacetylation of protein substrates (Vaziri et al., 2001) and PARP transfers ADP-ribose from NAD to itself and other nuclear proteins at sites of DNA single strand breaks to signal repair (Charron and Bonner-Weir, 1999). The potential for PARP to regulate NAD levels greatly outweighs that of Sirtuins, given that the later consume only one molecule of NAD per reaction while PARP has the capacity to generate polymers comprised of hundreds of ADP ribose moieties. Thus, we determined gene expression using qPCR and functional outputs of Sirtuin and PARP activities using a fluorometric enzyme assay and western blot of ADP-ribosylated proteins, respectively. While mRNA levels of both Sirt1 and PARP were elevated (Figure 2-4A), poly ADP-ribosylation was increased in FABP deficiency (Figure 2-4B) but Sirtuin-mediated deacetylation was not (reaction rates, 387.1±12.5 and 382.5±7.5, WT and aP2-mal1$^{-/-}$, respectively) (Figure 2-4C). Taken together, these observations signify that FABP deficiency evokes an increase in total nucleotide levels through transcriptional upregulation of the rate-limiting

NAD synthesis gene Nampt, while shifting the predominant species from NAD to NADH.

Citric acid cycle activation contributes to altered NAD/NADH ratio

NADH, typically produced during macronutrient catabolism, is a marker for carbon flux through the citric acid cycle, providing reducing equivalents to drive mitochondrial electron transport for ATP synthesis. Our data points to a nucleotide imbalance having potential effects on both substrate availability and substrate utilization. Indeed, there is a substantial impact on citric acid cycle pathway gene activation (Figure 2-5A) and a marked increase in the corresponding levels of citric acid cycle metabolites (Figure 2-5B). With the exception of citrate synthase and alpha-ketoglutarate dehydrogenase, all enzymes of the citric acid cycle are elevated between 1.5 to 2 fold, as determined by qPCR array (Figure 2-5A) Additionally, pathway intermediates were elevated between 1.5 and 4 fold (Figure 2-5B). Notably, all of the metabolites that directly participate in redox-reactions producing NADH were increased by several fold in aP2-mal$^{-/-}$ mice.

Although citric acid cycle activation provided a means to explain the high levels of NADH present in FABP deficient livers (Figure 2-3H), two fundamental questions remained. First, why has this switch in redox status occurred? Secondly, what impact does this alteration in nucleotide ratio have on liver metabolism?

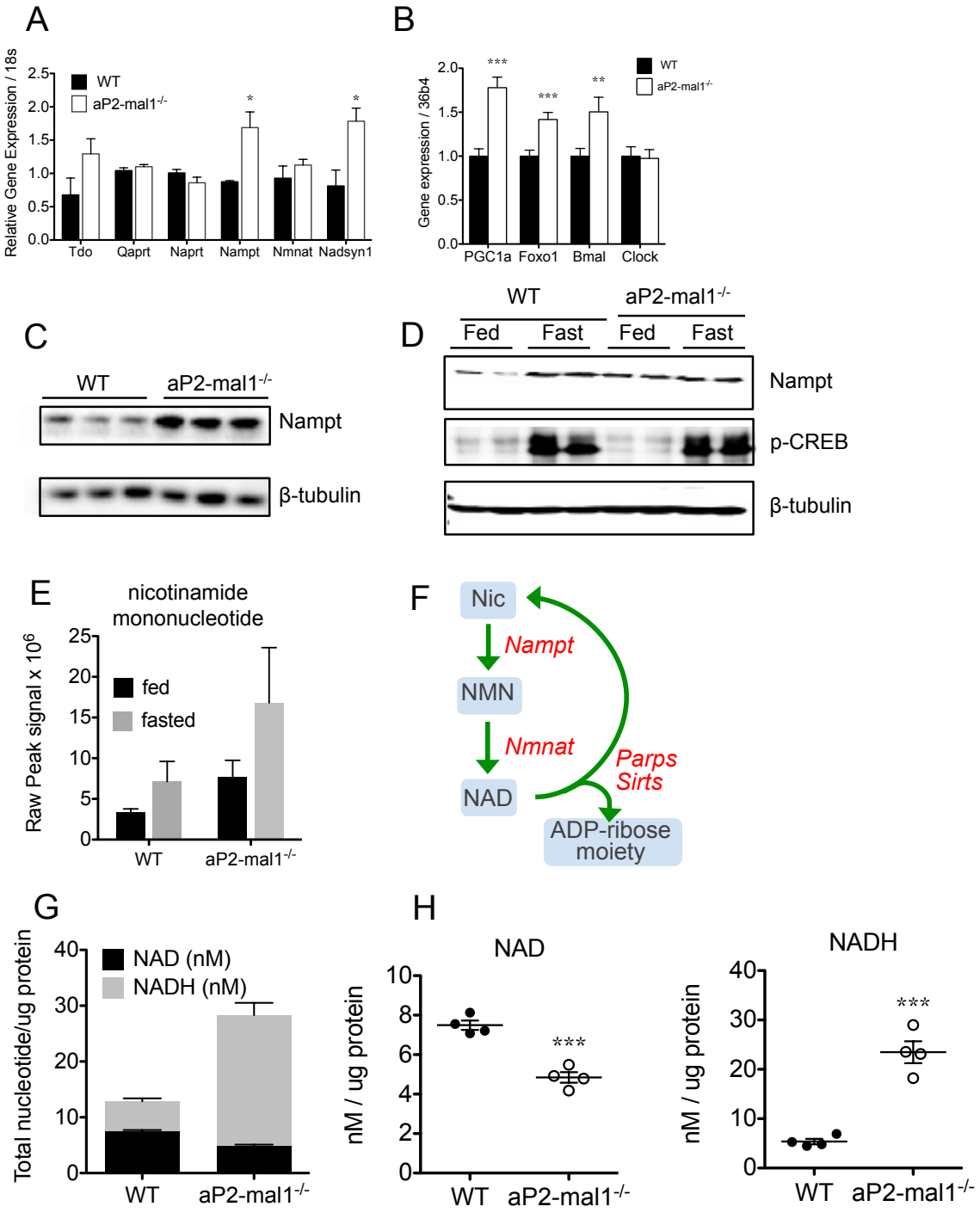

Figure 2-3. NAD metabolism in Liver.

Figure 2-3 (Continued). NAD metabolism in Liver.

(A) qPCR of genes encoding enzymes involved in each of the three pathways for NAD production and (B) transcription factors regulating Nampt. (C) N=4. (D) Immunoblot of rate limiting enzyme of NAD synthesis, Nampt in random fed animals, and (E) animals in the fed and fasted state. (E) Levels of nicotinamide mononucleotide, the immediate product of Nampt activity. (F) Graphical representation of NAD salvage pathway from nicotinamide. (G-H) Total nucleotide levels (G) as nanomolar ratios and (H) as separate species. Abbreviations: tryptophan dioxygenase (TDO), quinolinate phosphoribosyltransferase (Qaprt), nicotinamide/nicotinate mononucleotide adenylyltransferase (Nmnat), NAD synthetase (Nadsyn), nicotinic acid phosphoribosyltransferase (Naprt), nicotinamide phosphoribosyltransferase (Nampt). * $p<0.05$, ** $p<0.01$.

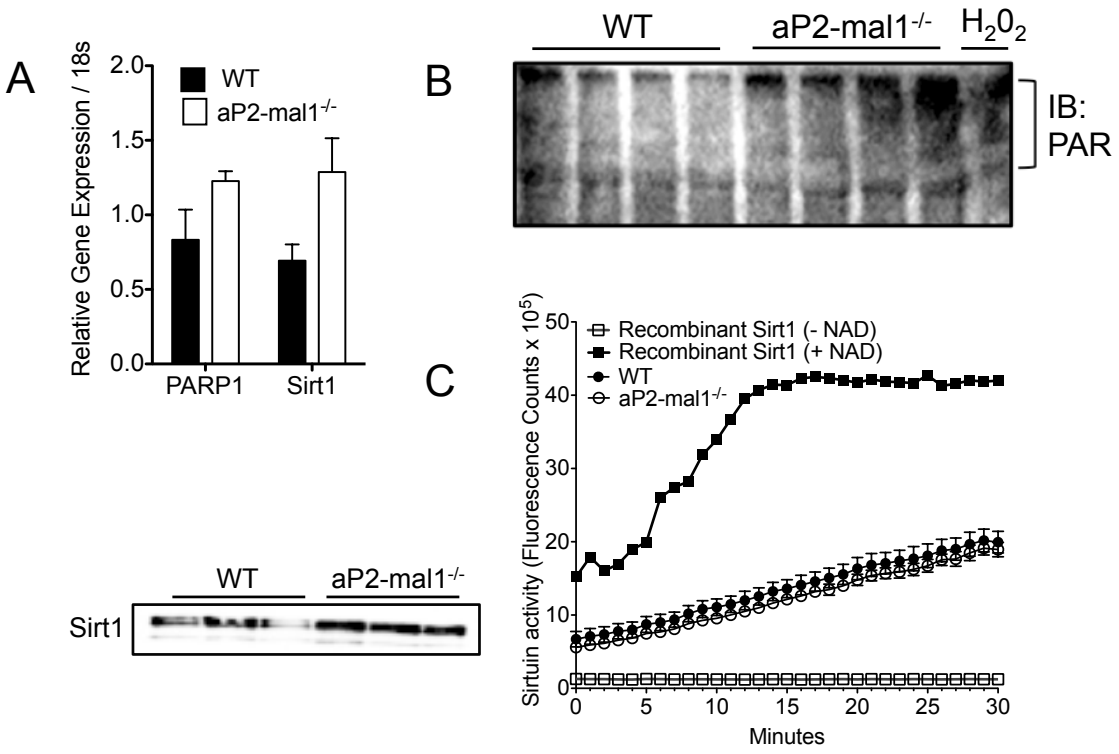

Figure 2-4. NAD consumption in FABP deficiency. (A) qPCR of NAD consuming enzymes, (B) Immunoblot of poly-ADP ribosylated proteins and an H_2O_2 treated HeLa cell lysate as a positive control. Line indicates range of 116-200kD corresponding to ribosylated proteins. (C) Sirtuin protein levels (left) and deacetylase activity (right) in 10 µg of liver lysate. Graph includes linear portion of 60-minute assay. Recombinant-Sirt1 was assayed +/- NAD as a positive control. N = 3-4, 12 weeks of age for each genotype.

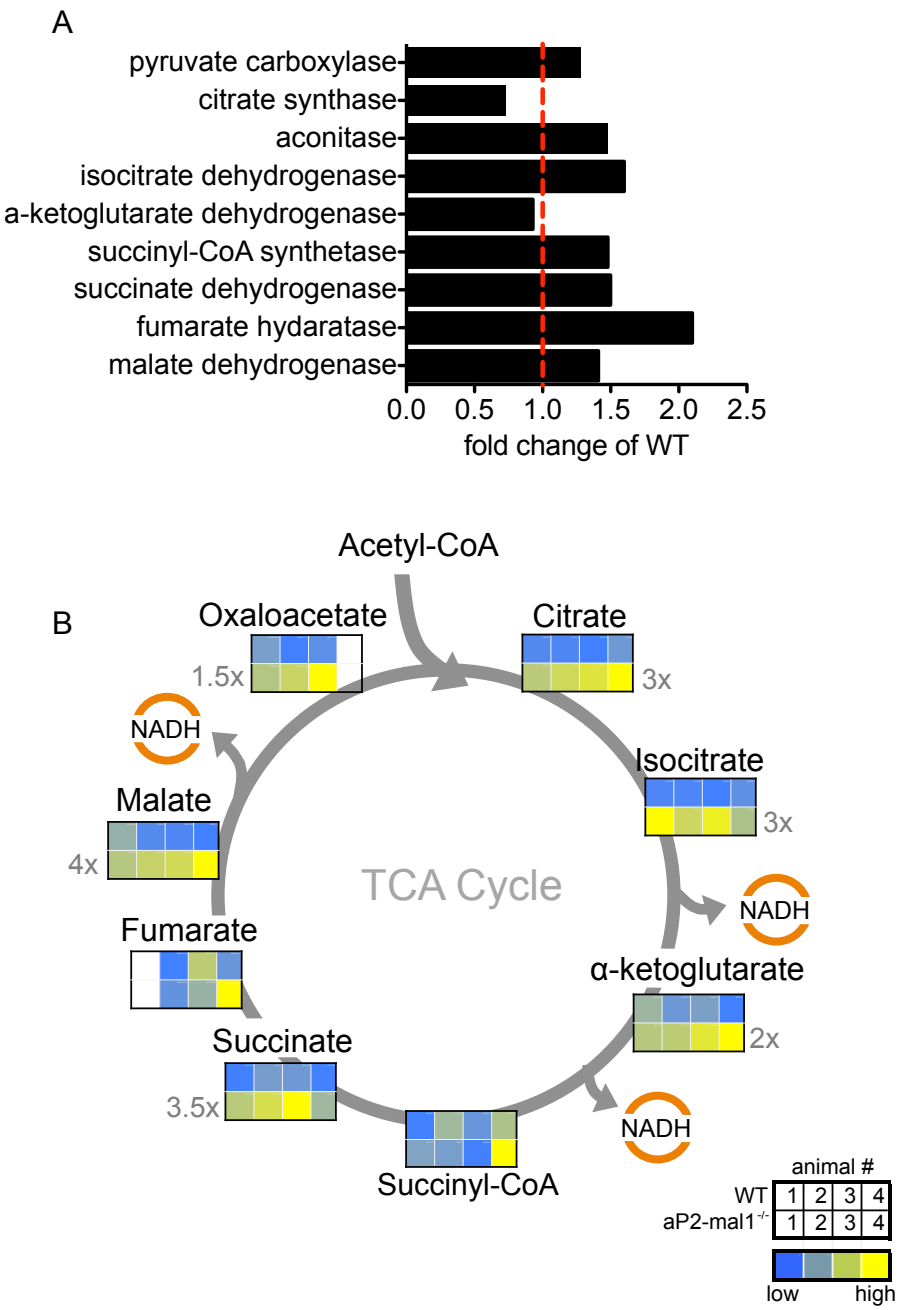

Figure 2-5. Citric acid cycle pathway activation. (A) Citric acid cycle gene expression as determined using SAB biosciences qPCR array on pooled cDNA from each genotype. (B) Heatmap of TCA cycle intermediates in aP2-mal1$^{-/-}$ versus wild type.

Metabolic reprogramming in FABP deficiency

Harvesting energy from nutritional substrates and stored macromolecules requires a series of steps that generate carbon units to be processed through the citric acid cycle. For small mammals, metabolic cage experiments are used to determine substrate utilization. In this type of experiment, O_2 intake and CO_2 produced from macronutrient catabolism are measured. From this, a respiratory exchange ratio (RER) is determined, representing the stoichiometric relationship between the amount of oxygen required and the amount of CO_2 produced in the complete oxidation of one mole of carbohydrate (RER = 1.0) or one mole of lipid (RER = 0.7). Using this standard method, we calculated the RER value over a 12-hour dark period. We found that there is a marked difference in the frequency distribution of RERs between wild type and aP2-mal1$^{-/-}$ mice across all intervals (Figure 2-6), with FABP deficiency causing a leftward shift towards lower RERs, suggesting an alteration of substrate preference from carbohydrates to fatty acids. [Determining substrate utilization from this value can however be complicated by two factors. First, highly glycolytic tissues release lactate into circulation leading to a rise in pH, necessitating the conversion of HCO_3 to CO_2 in a deacidification reaction. Having previously documented enhanced muscle glucose uptake in aP2-mal1$^{-/-}$ mice (Maeda et al., 2005), it seemed likely that these RER values could also reflect a measure of discordant CO_2 production in FABP deficient mice. Secondly, proteins cannot be completely oxidized to CO_2, thus it is difficult to attribute a single RER value to protein catabolism. However, given that protein catabolism is commonly

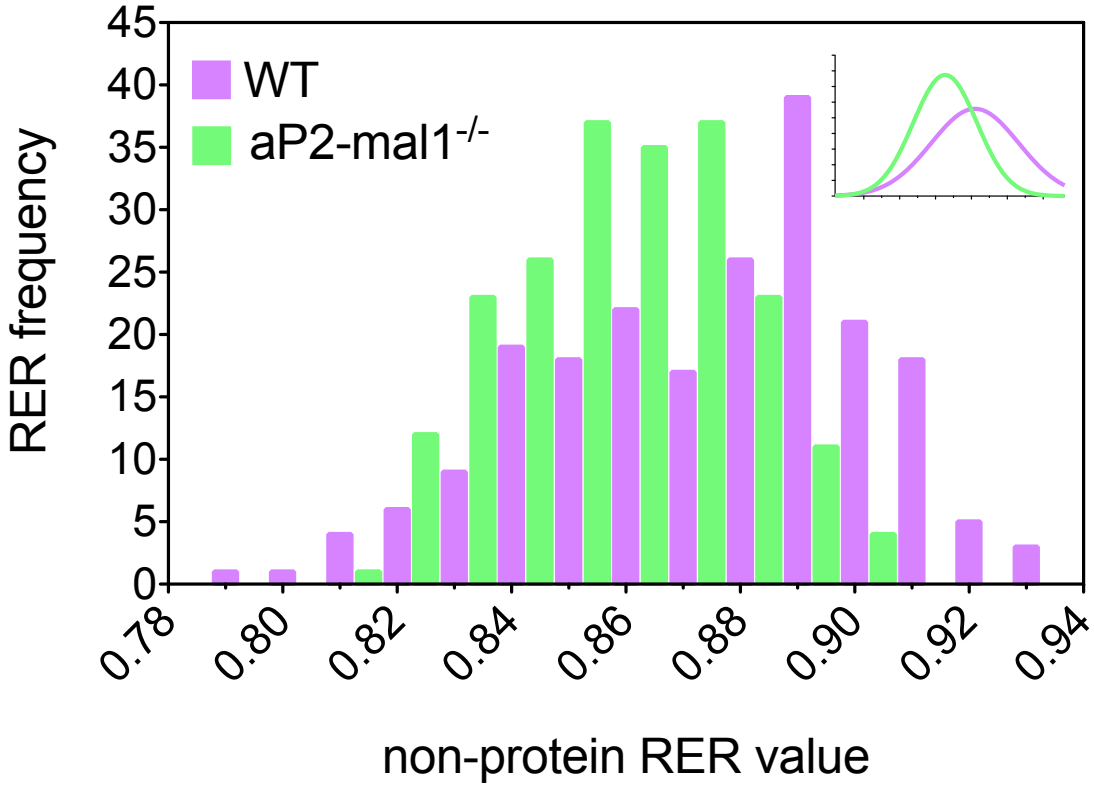

Figure 2-6. Evidence of altered substrate utilization in FABP deficiency.

Respiratory exchange ratios. VO_2 and VCO_2 gas exchange was measured over a 72-hour period. Plot represents frequency distribution of RERs recorded at 10-minute intervals over a 12-hour dark cycle. N = 4 per group.

regarded as a minor contributor to overall carbon flux under normal conditions, the inaccuracy associated with metabolic cage determination of substrate utilization, with respect to protein, is considered negligible and not accounted for. If indeed the case, blood buffering of lactate and/or activation of protein catabolism in FABP deficiency would lead to a significant overestimation of RER, and therefore, an underestimation of the fatty acid oxidation contribution to cellular respiration.

Given the caveats of metabolic cage monitoring, we explored the possible contribution of blood buffering and protein catabolism to RER in FABP deficiency. Lactate produced by highly glycolytic skeletal muscle is delivered to liver through the circulation to the liver, where it is metabolized. LC/MS analysis of lactate, however, revealed no apparent activation of this cycle in FABP deficiency, as skeletal muscle, plasma and liver lactate levels were comparable between both genotypes (Figure 2-7A). We then asked whether protein catabolism was activated in FABP deficiency by investigating the urea cycle, as ammonium released during protein deamination enters this pathway to be processed for excretion. While expression of ornithine transcarbamylase (OTC), arginosuccinate synthase 1 (ASS1), and arginosuccinate lyase (ASL) were all modestly elevated, OTC was the only urea cycle enzyme gene to reach a statistical increase in aP2-mal1$^{-/-}$ livers (Figure 2-7B and C). Additionally we found that Sirt3, which promotes urea cycle activity by deacetylation of OTC, was also modestly increased though not significantly (Figure 2-7B). (Hallows et al., 2011). Regarding activating metabolites of the urea cycle, N-

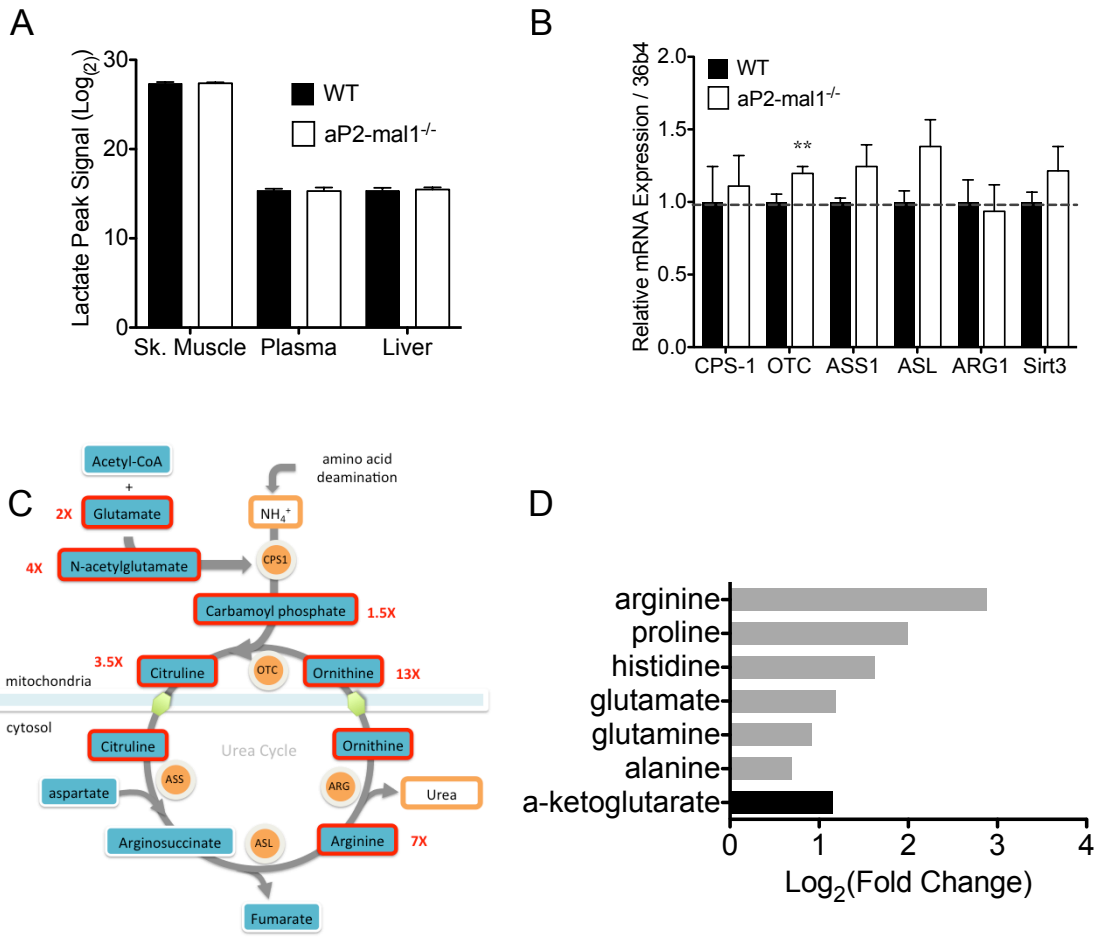

Figure 2-7. Activation of Protein Catabolism. (A) Lactate levels in skeletal muscle, plasma and liver, corresponding to the Cori cycle. (B) Gene expression of urea cycle enzymes. (C) Urea cycle intermediates increased in aP2-mal1$^{-/-}$ vs. WT are circumscribed by solid red line (≥ 1.5-Fold). (D) Amino acid mediated citric acid cycle anaplerosis. Fold Change (Log_2) indicates magnitude of increase in amino acids leading to production of α-ketoglutarate .

acetylglutamate and arginine were both increased in FABP deficient livers, by 4-fold and 7-fold, respectively (Figure 2-7C). Ornithine, another key intermediate in the urea cycle, achieved the highest fold increase (13-fold) of the 208 metabolites that were detected in liver, and was among several other enhanced cycle intermediates (Figure 2-7C). An important mechanistic feature of urea cycling, parallel to the detoxification of nitrogen generated from protein catabolism, is anaplerosis—the replenishment of citric acid cycle intermediates for the generation of NADH. Accordingly, all alpha-ketoglutarate related amino acids are present at high levels in aP2-mal1$^{-/-}$ livers (Figure 2-7D). This substrate-driven activation of the urea cycle underscores a dominant role for proteins in the replenishment of energy substrates in FABP deficiency. Adding further strength to this idea is the fact that pathways relevant to protein metabolism appeared high on both the metabolite enrichment and pathway impact scales, ranking adjacent to citric acid cycle, and nicotinamide metabolism (Figure 2-2A and B). Remarkably, though protein catabolism is known to predominate during high intensity exercise or prolonged fasting, FABP deficiency alone mimics the same pathway activation.

To determine the nature of this switch in substrate preference from carbohydrates to fatty acids revealed by the left-shift in RER frequency distribution, we examined glycolysis and fatty acid oxidation pathways. Glycolytic gene expression, protein levels of glucose transporter 2, and metabolites of glycolysis were all decreased in FABP deficient mice (Figures 2-8A,B,C). Conversely, expression of genes known to play a critical role in regulating mitochondrial

uptake of fatty acids and subsequent β-oxidation, CPT1α and Acadm, respectively, were each elevated by two-fold (Figures 2-8D and E).

Through this comprehensive analysis of the liver metabolome, we have confirmed enhanced activation of β-oxidation, and revealed a previously unknown phenotype of FABP deficiency: protein-mediated citric acid cycle anaplerosis. This alternative pathway activation likely contributes to an enhanced rate of NADH production, and improved homeostatic control when FABP deficiency is coupled with genetic or diet induced obesity. Together, these data allow us to assign a probable cause and source of the NAD/NADH nucleotide imbalance demonstrated in aP2-mal1$^{-/-}$ mice.

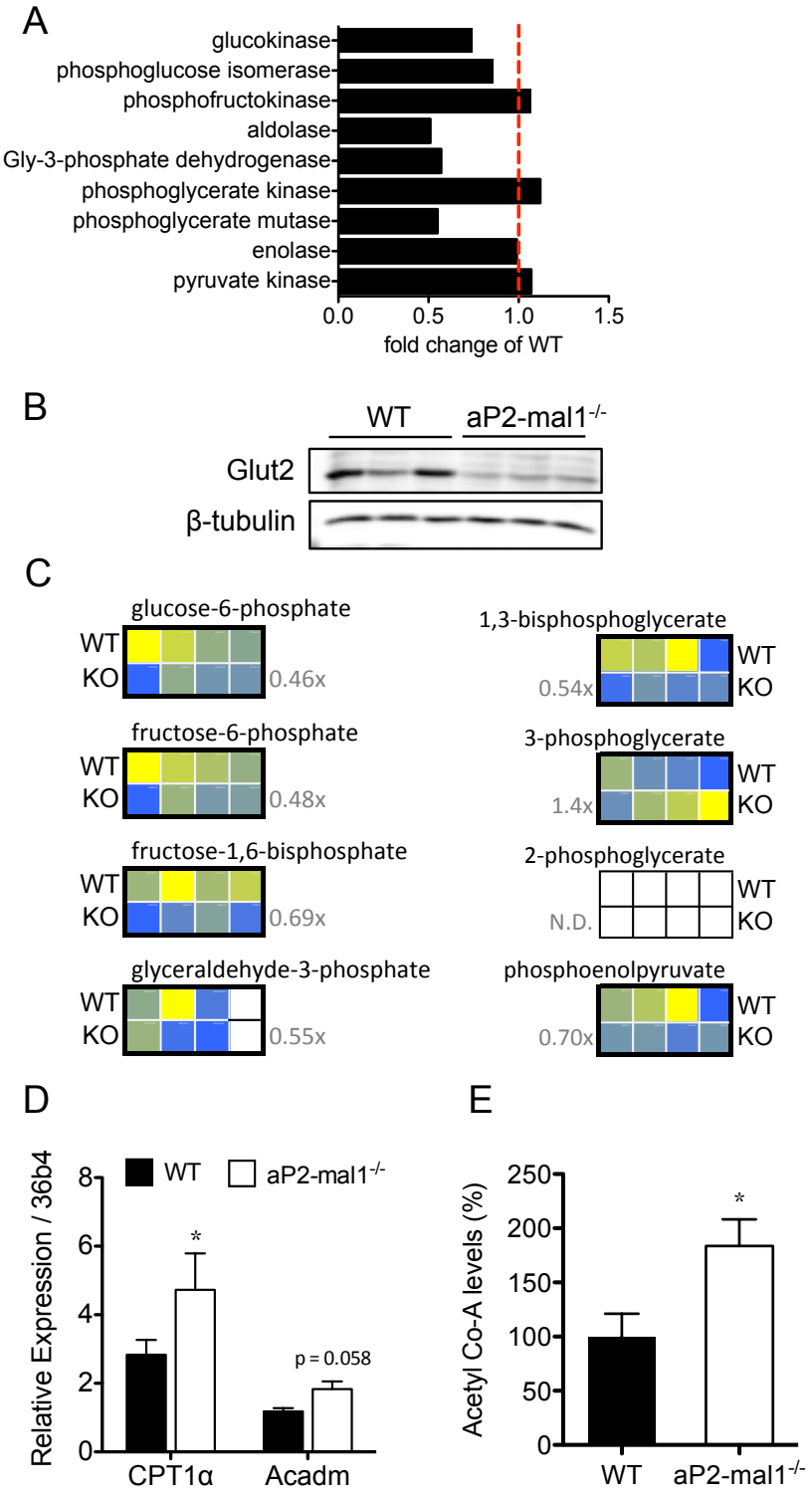

Figure 2-8. Metabolic shift from glucose utilization to lipid oxidation

Figure 2-8 (Continued). Metabolic shift from glucose utilization to lipid oxidation (A-C) Glucose metabolism. (A) Glycolytic gene expression as in figure 3A. (B) Glut2 protein levels and (C) heatmapping of glycolytic intermediates using same scale as figure 3b. Fatty acid oxidation. (D) Expression of critical genes for mitochondrial fatty acid uptake and oxidation, CPT1α and Acadm. (E) Acetyl-CoA levels.

aP2 influences substrate preference in hepatocytes

Since FAPB deficiency critically impacts substrate utilization in liver, and given that aP2 is a bona fide adipokine, we then asked whether aP2 itself could influence carbohydrate and/or lipid metabolism in a liver cell line. We first established that the effect of FABP deficiency on liver substrate utilization was non cell-autonomous, as primary hepatocytes isolated from wild type and aP2-mal1$^{-/-}$ mice responded similarly to a functional test of cellular respiration when exposed to glucose, palmitate, or glutamine (Figure 2-9A). Primary hepatocytes from the two genotypes also expressed comparable levels of Nampt (Figure 2-9B). Therefore, the effect of FABP deficiency on liver as demonstrated in Figure 2-3A is essentially lost in hepatocytes isolated from systemic metabolism.

We then pre-incubated Hepa1-6 cells with aP2 protein for 4 hours and then exposed them to either glucose or palmitate in the continued presence of aP2. Quite strikingly, aP2 increased glycolytic rate (Figure 2-10A) and decreased the rate of fatty acid oxidation (Figure 2-10B), both in a dose dependent manner. Recalling the effect of FABP deficiency on NAD/NADH levels, it seemed likely that these outcomes could be the consequence of an altered redox environment. Indeed, aP2 treatment moderately reduced total nucleotide levels (Figure 2-10C), and induced a dramatic shift of the ratio in favor of the oxidized form (Figure 2-10C). These data support the idea that aP2-mediated inhibition of fatty acid oxidation contributes to a substantial reduction in NADH and follows a model whereby FABP deficiency allows for higher rates of fatty acid catabolism and subsequent NADH production.

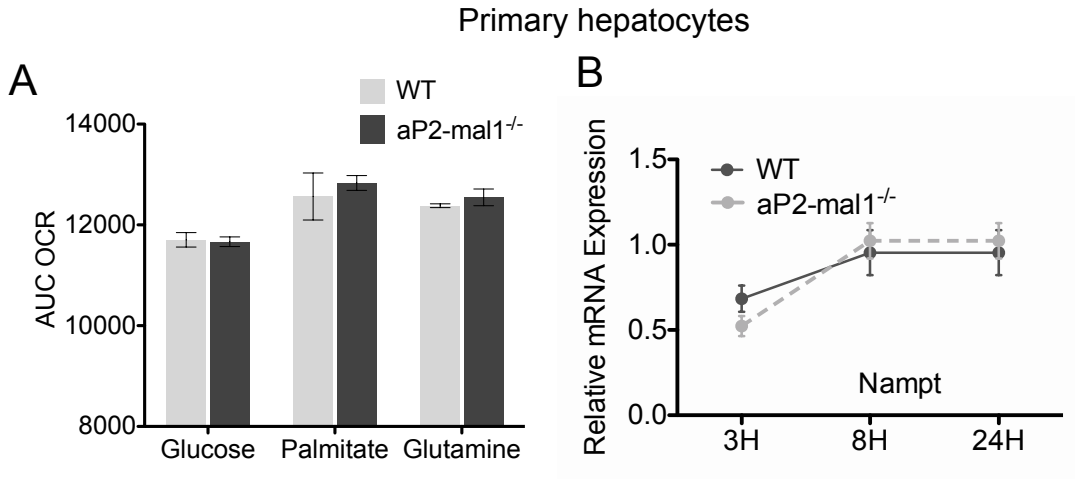

Figure 2-9. Cell non-autonomous regulation of liver substrate utilization.

Primary hepatocytes were isolated from wild type and aP2-mal1 deficient mice. (A) Cellular respiration was measured after exposure to energy substrates. (B) Expression of Nampt was measured at three time points after isolation.

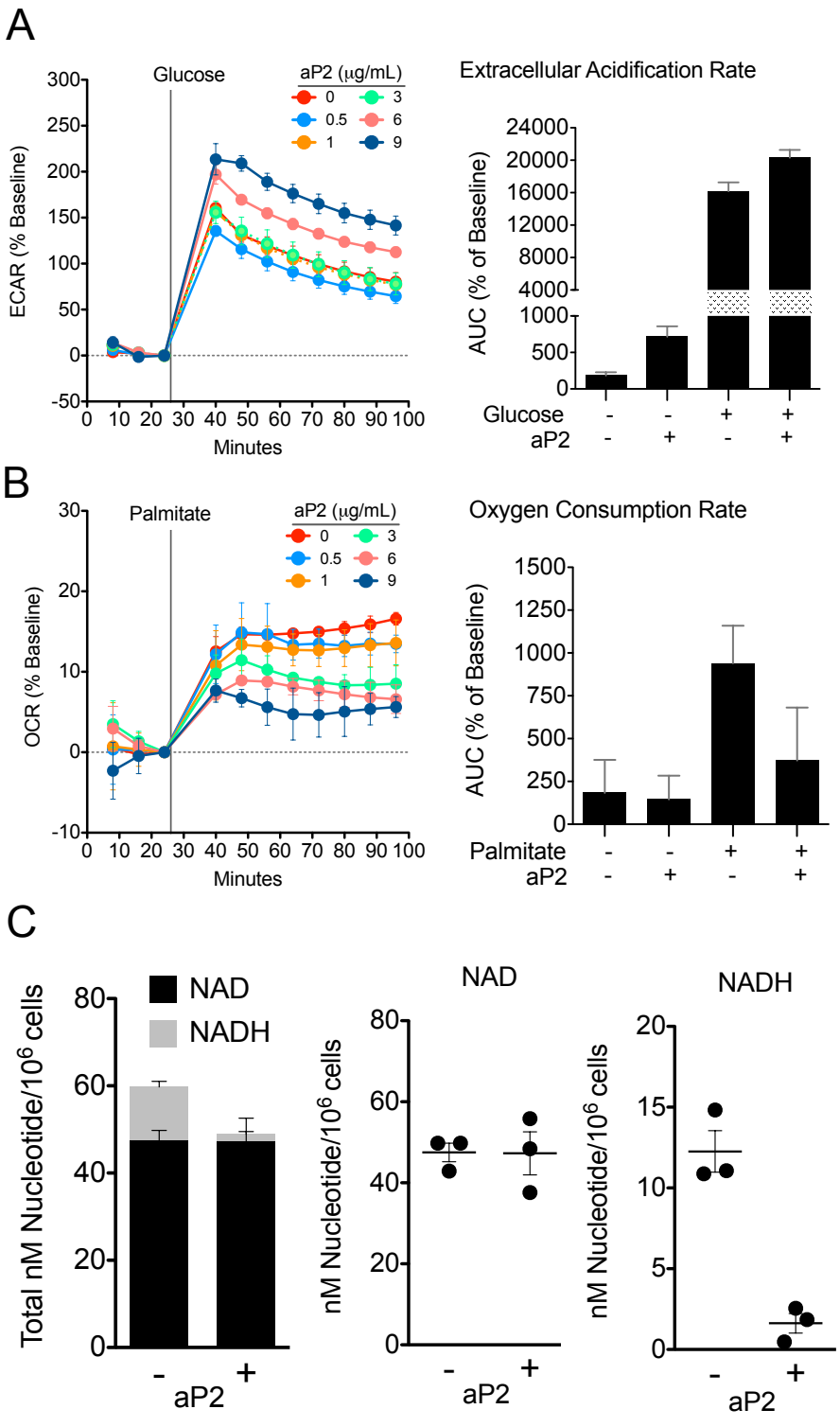

Figure 2-10. aP2 directs substrate utilization and alters nucleotide levels.

Figure 2-10 (Continued). aP2 directs substrate utilization and alters nucleotide levels. (A) Hepa1-6 cells pre-incubated with increasing concentrations of aP2. (A) Extracellular Acidification Rate (ECAR) is measured in response to glucose. (B) Oxygen Consumption Rate (OCR) is measured in response to palmitate. ECAR and OCR correspond to glycolytic rate and fatty acid oxidation, respectively. Graphs adjacent to 4A and 4B represent the area under the curve (AUC) for each measurement at the highest concentration of aP2 (9ug/mL) (C) Total nucleotide graphed as nanomolar ratios and separate species.

aP2 regulates hepatic Nampt in vitro and in vivo

Recently, Tao et al. reported that a liver specific knockout of FOXOs 1,3 and 4 exhibited hepatic steatosis that was accompanied by insufficient liver Nampt expression. Furthermore, this group demonstrated that adenoviral mediated overexpression of Nampt reversed this phenotype, as evidenced by decreased hepatic lipogenesis, a high rate of fatty acid oxidation and lower liver triglycerides. Taken together, these data suggest that Nampt modulates NAD levels in the regulation and maintenance hepatic lipid metabolism. As our findings suggest a role for FABPs in the regulation of nucleotide levels and fatty acid metabolism, we sought to experimentally determine a role specifically for aP2 in these processes.

To link aP2 function to the regulation of Nampt, we incubated Hepa 1-6 cells expressing a Nampt-luciferase promoter construct with mouse serum collected from wild type and aP2-mal1$^{-/-}$ mice. aP2-mal1$^{-/-}$ serum activated the Nampt promoter reporter and expression of endogenous Nampt protein to a greater extent than wild type (Figure 2-11A and B). These findings suggest that a component present in wild type serum suppresses Nampt transcription, and we hypothesized that the adipokine aP2 could play this role. To investigate this possibility, we employed the use of chemical (aP2i)- and antibody (aP2H3)- based inhibitors with demonstrated specificity for aP2 (Furuhashi and Hotamisligil, 2008). Both inhibitors of aP2 relieved the suppressive effect of wild type serum on Nampt promoter activity, which was restored to the same level as that measured in cells incubated with aP2-mal1$^{-/-}$ serum (Figure 2-11C).

To more specifically demonstrate that adipose tissue aP2 was responsible for this activity, we co-incubated Hepa 1-6 cells with differentiated adipocytes using the Transwell system. Again, we observed a repressive effect on Nampt transcription in cells co-cultured with adipocytes from mice with wild type levels of aP2, but not in cells co-cultured with adipocytes from aP2-mal1$^{-/-}$ mice (Figure 2-11D). Furthermore, we can recapitulate this negative regulation observed in the Hepa 1-6/WT adipocyte condition by addition of aP2 protein to the Hepa 1-6/AM$^{-/-}$ co-culture, leading to a deficiency in Nampt protein expression (Figure 2-11E). Lastly, we can counteract this effect, and restore Nampt protein levels by addition of aP2i or aP2H3 inhibitors (Figure 2-11E).

To support earlier findings that Nampt inhibition reduces fat oxidation, and to provide additional support for aP2 as an upstream negative regulator of Nampt, we employed a commonly used inhibitor of Nampt activity, FK866. As expected, both aP2 protein and FK866 inhibited palmitate oxidation, while dual exposure produced no added effect (Figure 2-12A and B). We also provided cells with the NAD pathway intermediates nicotinamide, and nicotinamide mononucleotide, which lie immediately up and downstream of Nampt activity, respectively. Hepa 1-6 cells were refractory to nicotinamide supplementation, however, the addition of nicotinamide mononucleotide reversed aP2-induced inhibition of palmitate oxidation (Figure 2-12C).

Finally, we could reliably recapitulate the effects of Nampt inhibition in vivo by intravenous administration of aP2 protein. After six hours of infusion, we were

able to detect a significant reduction in both Nampt mRNA and protein (Figure 2-11F and G). Furthermore, this effect was specific for Nampt, as there was no effect on gene expression of other key enzymes involved in NAD metabolism. This finding, together with in vitro data utilizing Hepa 1-6 cells exposed to mouse serum, co-cultured adipocytes, and recombinant aP2 solidifies aP2 as a negative regulator of Nampt expression (Figure 2-11H).

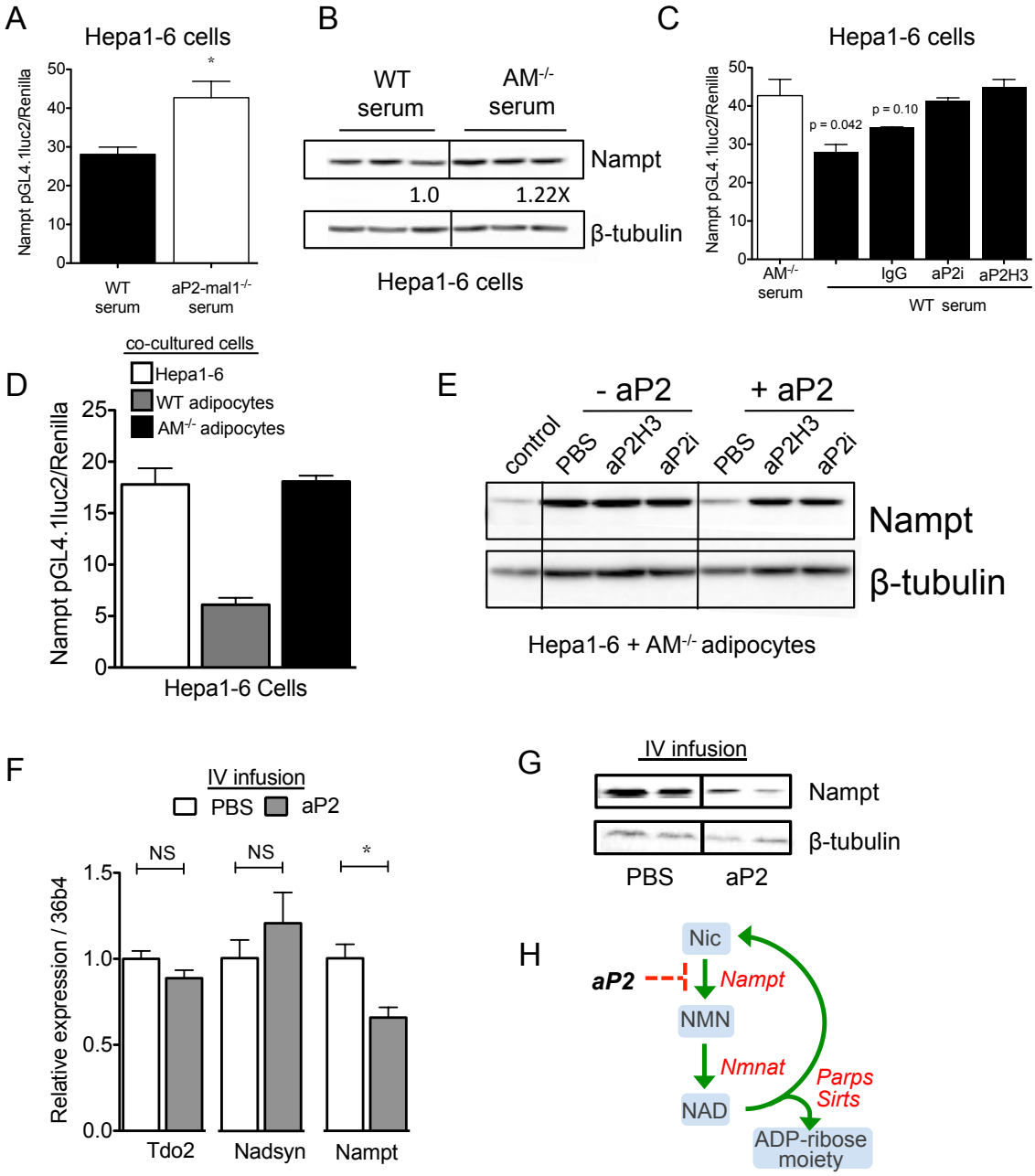

Figure 2-11. Suppression of Nampt by aP2 *in vitro* and *in vivo*.

Figure 2-11 (Continued). Suppression of Nampt by aP2 *in vitro* and *in vivo*. (A) Luciferase activity and (B) Nampt protein levels in Hepa 1-6 cells transfected with a Nampt-luciferase reporter construct exposed to either wild type or aP2-mal1$^{-/-}$ mouse serum. (C) Luciferase activity in cells treated in same manner as figure 5A and 5B with the addition of aP2 chemical inhibitor (aP2i) or aP2 antibody (aP2H3). (D) Hepa 1-6 cells co-cultured for 18 hours with differentiated adipocytes, or Hepa 1-6 cells as a control. (E) Nampt protein levels in Hepa 1-6 cells co-cultured with differentiated aP2-mal1$^{-/-}$ adipocytes exposed to aP2 protein in the presence and absence of aP2i and aP2H3. (F) Hepatic gene expression of NAD biosynthesis genes, and (H) protein levels of liver Nampt after 6 hour *intra venous* infusion of aP2 protein into aP2-mal1$^{-/-}$ mice.

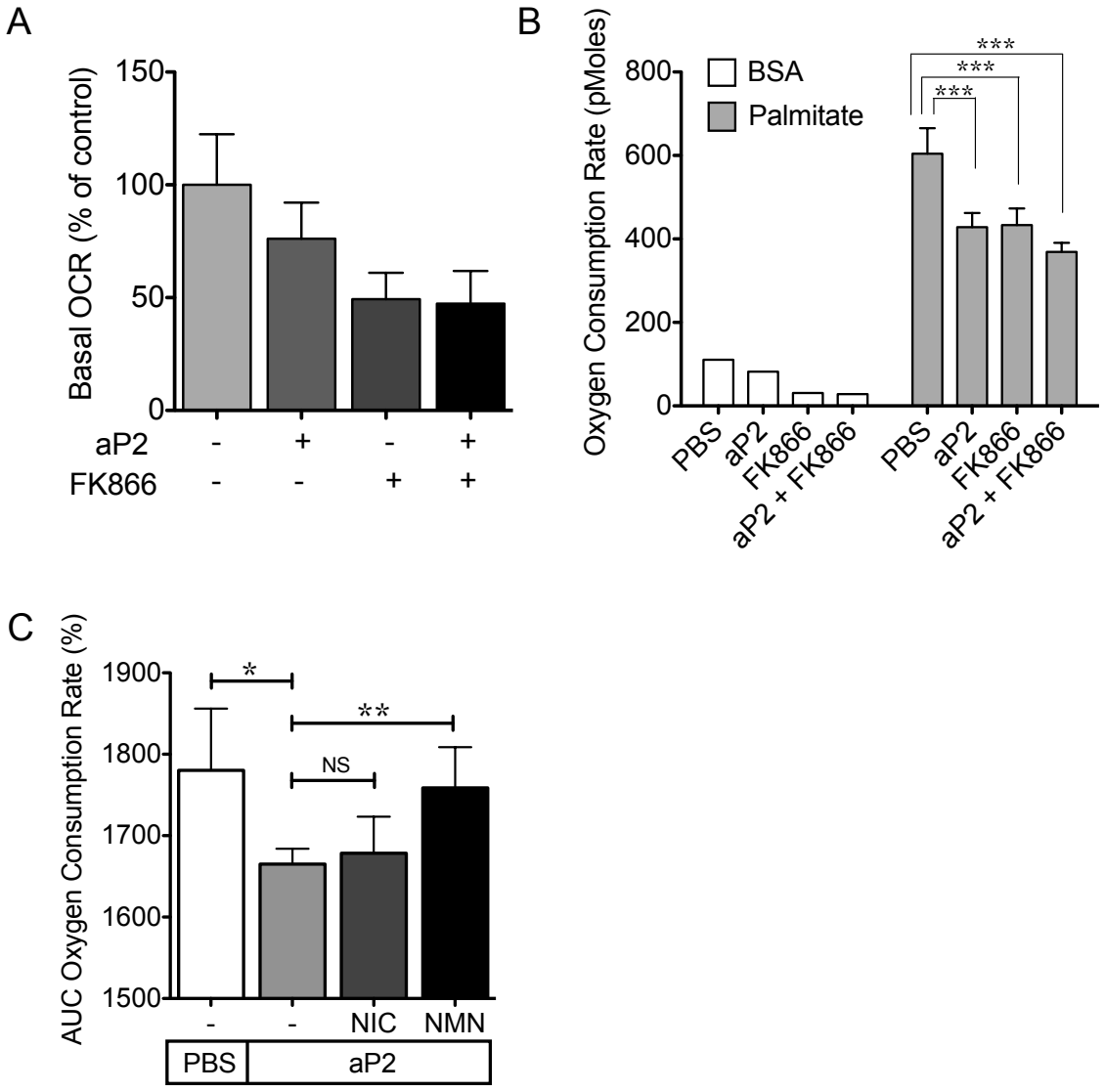

Figure 2-12. Inhibition of palmitate oxidation by aP2 and small molecule inhibitor of Nampt. (A-B) OCR in Hepa 1-6 cells exposed to aP2 protein or FK866 for 4 hours. (C) Area under the curve of OCR after exposure to aP2 in the presence of NAD pathway metabolites.

DISCUSSION

Besides achieving satiety, the major purposes of food intake are to provide sufficient sources of carbon for the generation and maintenance of biological structures, and to meet the variable need for storage and supply of energy. De novo synthesized lipids, carbohydrates, and proteins, accompanied by a milieu of metabolites and hormonal signals all together comprise a critical and complimentary exchange mechanism between intrinsic factors and dietary inputs.

Hormones allow for adaptation to acute perturbations in substrate levels. For example, insulin secreted in response to increased plasma glucose directs its uptake into peripheral tissues, and liver responds by activating glycolytic and lipogenic transcriptional programs. On the other hand, sustained alterations in substrate availability or utilization capacity challenge the ability of an organism to meet energy demands. We have previously shown that a combined deficiency of aP2 and mal1 protects from hepatic steatosis and improves glucose homeostasis by down-regulating the lipogenic and glucogenic programs in liver (Maeda et al., 2005, Cao et al., 2013). Given that these pathways are inextricably linked to the storage and utilization of energy required for normal cellular maintenance, we aimed to understand the impact of these regulatory changes on hepatic metabolite flux. One limitation of mechanistic studies involving metabolic phenotypes is an incomplete knowledge of which substrates are available for catabolism and whether there is preferential usage among those available substrates. As is the custom in the field, we regularly assess substrate availability by

measuring concentrations of substrates most proximal to energy production (i.e., glucose and major classes of lipids) and perhaps link data with respiratory exchange ratios determined from metabolic cage experiments to determine utilization. These data however, are only a fractional representation of metabolite flux and extrapolations towards substrate utilization from these experiments lack sufficient resolution to address complex phenotypes. Given the centrality of lipids to metabolism and the ability of fatty acid binding proteins to influence multiple levels of systemic regulation, we sought to provide a comprehensive profile of metabolites altered in FABP deficiency using metabolomics. LC/MS revealed a dramatic reprogramming of metabolite flux in liver as evidenced by the enrichment of metabolites associated mainly with nicotinamide metabolism, amino acid deamination and ammonia detoxification. Although usually considered to be relevant only under prolonged fasting conditions, proteins can be mobilized to generate energy substrates by liberating glucogenic, ketogenic, and anaplerotic amino acids. To achieve this, the liver forms alpha-ketoacids by the deamination of proteins, and utilizes the resultant carbon skeletons to generate acetyl-CoA, pyruvate, or citric acid cycle intermediates. We suggest that this process is initiated to fuel citric acid cycle intermediates in response to a dampening of glycolysis and increased acetyl-coA produced from fat oxidation. Moreover, we provide evidence that that urea cycling contributes mainly to the replenishment of citric acid cycle intermediates for the production of NADH.

The constitutively high levels of liver Nampt protein and hepatic nucleotide imbalance we observe each bear a remarkable resemblance to dietary restriction, an intervention known to extend healthspan and longevity in a variety of organisms, including primates (Colman et al., 2009). The adaptive response to dietary restriction involves suppression of glycolysis, enhanced liver gluconeogenesis and glucagon-stimulated degradation of glycogen stores in order to maintain glucose levels. Over time, insufficient availability of carbohydrates will stimulate lipolysis and trigger the mobilization of lipids from adipose tissue (Dhahbi et al., 2001). Upon depletion of fat stores, the organism is then forced to catabolize muscle protein for use as an energy substrate. Remarkably, ad libitum fed mice deficient in FABPs are able to access pathways associated with long term dietary restriction, yet, are resistant to the wasting of skeletal muscle and peripheral tissue that occurs during starvation (Gabriely and Barzilai, 2001).

A key defining feature of FABP deficiency, also mimicking dietary restriction, is reduced adipose tissue mass, a feature that becomes even more pronounced when mice are challenged with a high fat diet. (Hotamisligil et al., 1996; Maeda et al., 2005). We propose a model whereby FABP deficiency allows for more effective utilization of fat by hepatocytes. High levels of β-oxidation, and the nearly two-fold increase in acetyl-CoA, each provide an abundance of carbon units for entry into the citric acid cycle. We found that in order to accommodate entry of large amounts of acetyl-CoA, citric acid cycle anaplerotic mechanisms were activated in FABP deficiency, marked by a substantial increase in urea cycle intermediates and

elevated anaplerotic amino acids. Our previous studies indicate that gluconeogenesis, a pathway that requires NADH, is suppressed livers of aP2-mal1$^{-/-}$ mice. It is likely then, that the coupling of decreased gluconeogenesis with increased citric acid cycle activity favors high NADH. Consistent with this reduced-state cellular environment in FABP deficiency was an inhibitory effect on the glycolytic pathway. Together, these findings demonstrate that FABP deficiency somewhat mimics dietary restriction by permitting more efficient fat utilization while also diminishing the activity of glucose-dependent energy generating pathways. Moreover, we demonstrate the ability of adipose derived aP2 to engage biochemical pathways in hepatocytes that direct substrate utilization resulting in an increased rate of glycolysis, and suppression of fatty acid oxidation. Our data indicates that FABPs conduct a major role in the communication of nutritional status between adipose and liver, and that their absence contributes to reprogramming of hepatic substrate metabolism in a manner that mimics the effects fasting.

It is well known that NAD cycles between both oxidized and reduced forms during cellular energy metabolism, though more recently it has also been shown to be consumed by reactions that transfer the ADP-ribose moiety to additional targets, or by enzymes that couple NAD cleavage to their catalysis (Mattevi, 2006). The existence of a second regulatory role for NAD underscores the potential influence of not only NAD biosynthesis enzymes, but also of NAD consuming enzymes on glucose utilization, fatty acid metabolism, and subsequent substrate

transformations involving redox reactions. Through our *in vitro* modeling of an adipo-hepatic regulatory axis utilizing mouse serum, liver cell/adipocyte co-culture, we have established aP2 as a negative regulator of Nampt expression (Figure 5H). Infusion of aP2 protein into aP2-mal1$^{-/-}$ mice confirmed that this regulatory model also applies to metabolism *in vivo*.

The emerging facts indicate that the benefits of longevity result from a delayed development of age-related diseases in mammals, many of which have known causal links to metabolism such as type 2 diabetes, cancer and cardiovascular disease (Hursting et al., 2001; Lane et al., 1999; Manco and Mingrone, 2005). Despite much attention surrounding the life extending effects of dietary restriction, the factors mediating the relationship between a restrictive diet and postponement of these outcomes remain largely elusive. We have previously shown protection against the deleterious effects of high-fat diet in FABP deficiency, including resistance to hepatic steatosis accompanied by a substantial increase in muscle glucose uptake (Cao et al., 2008). Here we demonstrate that genetic deficiency of aP2 re-directs substrate utilization towards fatty acid oxidation rather than glycolysis, thus opposing the canonical energy substrate preference of liver. It is therefore likely that the protective phenotype demonstrated by FABP deficient mice on high fat diet is due, at least in part, to a heightened capacity for fatty acid oxidation.

Our current findings, together with previous observations validate the designation of aP2 as a desirable therapeutic target—An adipocyte derived

circulating factor that exhibits a specialized role in regulating hepatocyte function while having minimal impact on the metabolome at additional sites in the periphery.

MATERIALS AND METHODS

aP2-mal1$^{-/-}$ mouse model

Mice with combined aP2-mal1 deficiency were generated from an intercross between C57BL/6J-aP2$^{-/-}$ and C57BL/6J-mal1$^{-/-}$ mice (Maeda et al., 2005). Both aP2 and mal1 expression were undetectable in adipose tissue depots. Furthermore, there was no compensatory upregulation of other FABP isoforms in adipose tissue of aP2-mal1 deficient mice.

Targeted Liquid-Chromatography Mass Spectrometry (LC/MS)

100 mg of tissue samples were homogenized in 1ml of 80% v/v methanol. Insoluble material in lysates was centrifuged at 14,000rpm for 15 minutes and resulting supernatant was evaporated using a refrigerated speed-vac. Samples were re-suspended using 20uL HPLC grade water for mass spectrometry at Beth Israel Deaconess Medical Center mass spectrometry core facility. 10µL were injected and analyzed using a 5500 QTRAP triple quadruple mass spectrometer (AB/MDS Sciex) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 249 endogenous water soluble metabolites for analyses of samples. Some metabolites were targeted in both

positive and negative ion mode for a total of 298 SRM transitions. ESI voltage was 5000V in positive ion mode and –4500V in negative ion mode. The dwell time was 5 ms per SRM transition and the total cycle time was 2.09 seconds. Samples were delivered to the MS via normal phase chromatography using a 2.0 mm i.d x 15 cm Luna NH2 HILIC column (Phenomenex) at 285 µL/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5 minutes; 42% B to 0% B from 5-16 minutes; 0% B was held from 16-24 minutes; 0% B to 85% B from 24-25 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate in 95:5 water: acetonitrile. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v1.1 software (Applied Biosystems). Glucose-13C labeled samples were run with 249 total SRM transitions (40 in positive ion mode and 209 in negative ion mode) with a total cycle time of 0.464 seconds. Data processing, statistical analysis, and pathway mapping was performed using the internet based platform, MetaboAnalyst (Xia and Wishart 2011).

Quantitative PCR and Western blotting

Total RNA was isolated from 100 mg of tissue using Trizol reagent. cDNA synthesis was performed using BioRad 5x iscript supermix. SYBR green based qPCR was performed on an ABI Thermocycler Primer sequences available as supplementary information. Western blots were prepared from SDS-PAGE gels as previously

described. Polyclonal antibody anti-Nampt (PBEF) (abcam ab45890). Monoclonal anti-PAR Polymer antibody (BD Pharmingen). Glut2 (Santa Cruz SC-9117). Sirt1 (Millipore 07-131). β-tubulin HRP (abcam ab21058).

NAD/NADH nucleotide levels

Livers were collected from exsanguinated mice was homogenized in either NAD or NADH extraction buffers—30 mg tissue each. Nucleotide concentrations were determined using a fluorescent NAD/NADH detection kit according to manufacturer's protocol (Cell Technology, Inc # FL NADH 100-2).

Glycolysis and Fatty Acid Oxidation measurements

Extracellular acidification rate and oxygen consumption rate were measured in Hepa 1-6 cells using the XF96 extracellular flux analyzer from Seahorse Bioscience. Cells were seeded at a density of 10K cells per well one day prior to assay in complete media. For the assay, cells were exposed to XF assay media supplemented 5mM glucose and 1% serum. OCR values represent Palmitate OCR minus BSA OCR.

Cell culture studies

Hepa1-6 were cultured in DMEM supplemented with 10% CCS and antibiotics. Nampt-luciferase reporter construct was a kind gift from the laboratory of Charlie Dong (Indiana University). Mouse blood was collected from FABP-deficient mice after 6-hour daytime food-withdrawal, using terminal blood collection by cardiac

puncture. After 20-30 minutes of clotting time on ice, samples were centrifuged for 20min at 8000 rpm, 4^0C. After removal of clotting factors, serum will be collected and stored at –80^0C. Mouse serum was pooled from 8 mice and supplemented at a concentration of 18%. Preadipocytes were differentiated using standard methods.

Sirtuin deacetylase activity

Activity measurements were made using Cyclex Sirt1 deacetylase fluorometric assay kit according to manufacturers protocol. (Cyclex, CY-1151). Briefly, Livers were homogenized in 10-fold volume Modified RIPA Buffer minus protease inhibitors. Using a 50µl reaction volume, appearance of the deacetylated fluorescent substrate was measured at one-minute intervals for 30 minutes using SoftMax Pro microplate reader at excitation and emission wavelengths of 340 and 440nm, respectively. Assay Buffer: 50mM Tris-HCl, pH8.8, 4mM MgCl2, 0.5mM DTT, 50X Fluoro-Subsrate Peptide, 5mAU/mL lysyl endpeptidase, 10X Trichostatin A, 2mM β –NAD.

Primary hepatocyte isolation

Mice were anesthetized using a ketamine/xylene mixture, and perfused through the hepatic portal vein with 20 mL of Hank's Balanced Salt Solution (HBSS) buffer warmed to 40^0C at a rate of 1.8 mL/minute. Following this, the liver is perfused with 20 mL of HBSS buffer containing collagenase (HBSS+) at the same rate. The liver was excised and transferred to a petri dish containing 5 mL of HBSS+ on ice, and cells were dispersed using forceps. This cell suspension was transferred to a tube

containing 35 mL of DMEM + 10% calf serum and subsequently filtered through a 70 µM cell strainer. The hepatocytes were centrifuged at 500rpm for 1 minute, washed once with DMEM and centrifuged again at 500 rpm for 1 min. The cell suspension was overlaid with 40% percoll in PBS and centrifuged at 800rpm for 10 min. Cells were re-suspended in William's E media and plated onto collagen coated plates.

Intra venous aP2 infusion

Mice with combined aP2-mal1 deficiency were reconstituted with aP2 by infusing recombinant protein into a jugular vein catheter at a dose of 8µg/kg/min. *Intra venous* aP2 infusion was performed under pancreatic clamp conditions to determine the ability of aP2 to directly influence Nampt gene expression.

Statistical analysis

Unless otherwise described, statistical significance testing was performed using Student's t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are presented as mean ± SEM for each group.

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CHAPTER 3

A ROLE FOR FABPs IN HEALTHSPAN AND LONGEVITY

BACKGROUND

Extended lifespan in rodents subjected to dietary restriction was first observed nearly eighty years ago (McCay et al., 1935). Since this discovery, efforts have focused on characterization of the physiological parameters that are altered under this regimen, identification of genes required for the metabolic shift that occurs during caloric restriction, and their subsequent mechanistic role. To date, dietary restriction has proven to be the most reproducible way to increase lifespan in animals, and it is speculated that the major factor regulating longevity in these animals is the diminution of white adipose tissue, perhaps through changes in secretion of adipose specific hormones that have peripheral effects on systemic metabolism.

A key defining feature of FABP deficiency is reduced adiposity. Barzilai et al. have demonstrated reversal of hepatic insulin resistance and aging in rodents under dietary restriction and suggest that a reduction in visceral adipose is the chief underlying determinant. Additionally, *C.elegans* FABPs have been identified in RNAi based genetic screens performed by two separate groups as putative longevity related genes (Ha et al., 2006). First by proteomic analysis of genes involved in cellular senescence (Ha et al., 2006) and secondly, in a screen performed in lipid deficient *C. elegans*, the worm homologue of aP2 was identified among 16 other potential candidate genes that affect fat storage and longevity (Wang et al., 2008).

Our present finding that circulating aP2 directs NAD metabolism in hepatocytes along with several previous studies linking NAD metabolism to the positive health effects observed in DR (Imai, 2009) further suggest a critical role for adipose tissue in regulating healthspan. To explore this potential, we aimed to determine if the lack of lipid chaperone action in adipose tissue mimics DR induced modifications in lipid pools, gene regulatory networks, peripheral tissue function and long-term survival.

RESULTS

Adipose tissue alterations in FABP deficiency and dietary restriction

Our lab has shown that mutations in adipose tissue fatty acid binding proteins aP2 and mal1 leads to increased production and secretion of a newly identified lipid hormone, palmitoleate. This fatty acid was demonstrated to link peripheral tissues in an endocrine network and exert positive effects on metabolic homeostasis. Furthermore, palmitoleate is a robust marker of *de novo* lipogenesis. Interestingly, the FABP-mediated changes in adipose tissue lipid composition, and the marked increase in *de novo* lipogenesis preceding this effect, are both highly reminiscent of dietary restriction induced adaptations in adipose tissue (Varady et al., 2007). Through comparative analysis of individual lipid classes and fatty acid species, we identified lipids that are present at similar levels in adipose tissue of DR and FABP-deficient mice. We found that FABP deficiency and dietary restriction both led to

similar degrees of elevation in fatty acid content in the free fatty acid and diacylglycerol lipid fractions (Figures 3-1A and B). Higher levels of diacylglycerol are correlated with lowering of plasma TGs, increased energy expenditure, and reduced adiposity (Murase et.al. 2001), and increased adipose tissue free fatty acids generated by *de novo* lipogenesis have proven to be beneficial as a means to produce palmitoleate (Cao et.al. 2008). When comparing changes that occur in both FABP deficiency and dietary restriction, palmitoleate levels were increased by approximately 65-80% in three major lipid classes (Figure 3-1C).

After verification that critical constituents of the lipid environment were regulated in the same manner by both FABP deficiency and dietary restriction, we further queried the molecular underpinnings of this phenotype by contrasting the transcriptional profiles of adipose tissue from animals subjected to each of these interventions with their respective controls. Compared to *ad libitum* fed control mice, animals on a long-term restricted diet had several hundred genes related mainly to carbohydrate and lipid metabolism that were significantly altered in adipose tissue. We then derived a comparison between gene expression profiles of DR mice and the transcriptional alterations that occurred in aP2-mal1 deficient mice when compared with their wild type controls. We determined that the regulatory overlap included 57 genes (Figure 3-2). This set included genes encoding factors related to amino acid, carbohydrate, lipid and mitochondrial

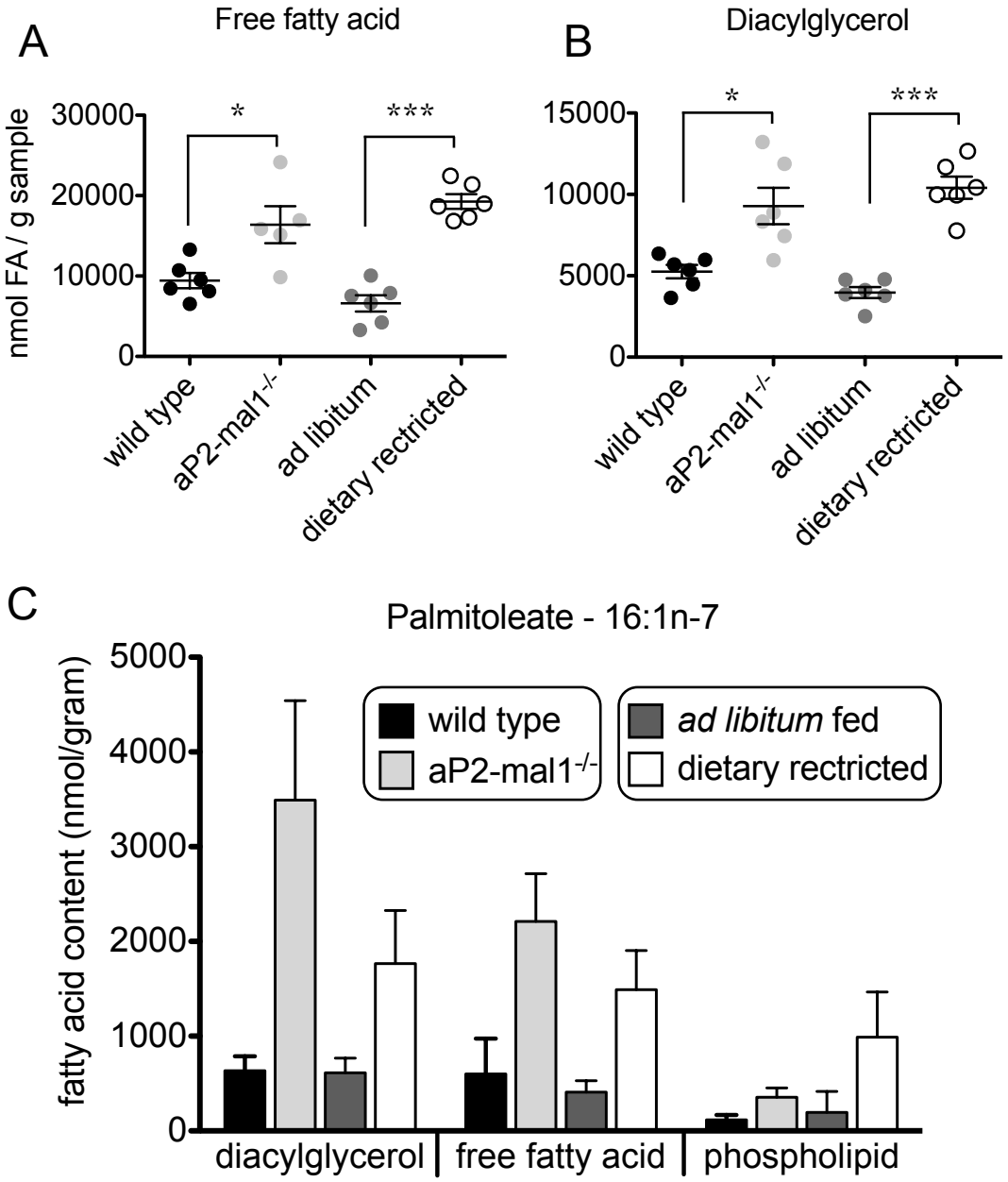

Figure 3-1. Trends in adipose tissue lipid profiles between adult male aP2-mal1$^{-/-}$ and dietary restricted mice.

Figure 3-1 (Continued). Trends in adipose tissue lipid profiles between adult male aP2-mal1$^{-/-}$ and dietary restricted mice. (A) Fatty acid content (nmol/g) in free fatty acid and diacylglycerol lipid pools and (B) palmitoleate levels in adipose tissue of aP2-mal1$^{-/-}$ vs. wild type mice and dietary restricted vs. *ad libitum* fed mice. One-way ANOVA and Bonferroni's multiple comparison test was used to determine significance and illustrate trends within each major lipid class (C). Percent increase in quantitative levels of palmitoleate in each lipid class was between 65-82% in each lipid class.

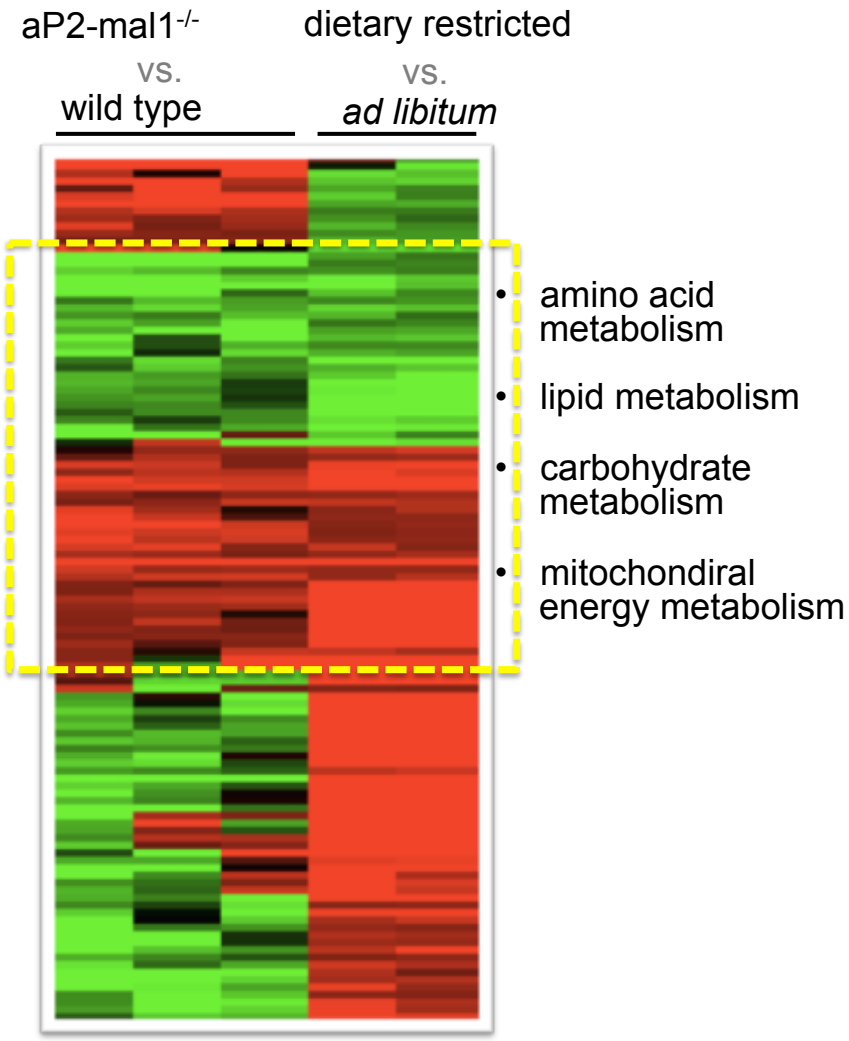

Figure 3-2. Comparison of gene expression profiles in adipose tissue of aP2-mal1$^{-/-}$ vs. wild type mice and dietary restricted vs. *ad libitum* fed mice.

Common transcriptional responses were determined using in house data obtained from FABP-deficient mice generated in our laboratory and publicly available microarray data from dietary-restricted mice (NCBI GEO datasets GSE 11845). Yellow box indicates 57 genes that are similarly altered by both FABP deficiency and dietary restriction.

energy metabolism; establishing that the phenotypic correlations between these interventions extend beyond qualitative changes in lipid content, towards deeper molecular parallels, as demonstrated by highly comparable gene expression of key metabolic genes such as GLUT4, glycerol-3-phosphate dehydrogenase, fatty acid synthase, ATP-citrate lyase, and several mitochondrial enzymes.

These observations implicated a role for adipose tissue fatty acid binding proteins in replicating the improved metabolic outcomes displayed during DR, possibly serving as an integrative link between the metabolic outcomes and increased survival that result from a DR regimen. Taken together, this cadre of physiologal correlates, parallels in adipose tissue lipid environment, and overlap in transcriptional regulation between FABP knockouts and mice subjected to the lifespan extending DR intervention provided a suitable rationale for conducting a long-term survival study in aP2-mal1$^{-/-}$ mice.

Long-term survival

We endeavored to determine if the lack of FABP action in adipose tissue mimicked the pro-health and pro-longevity benefits of dietary restriction. For this, we generated littermate cohorts of 40 wild type and 40 FABP-deficient mice of each gender (160 total) and allowed them to live out their normal lifespan. Surprisingly, there was a remarkable closeness in survival curves of female wild type and aP2-mal1$^{-/-}$ mice, and no degree of statistical difference in either gender.

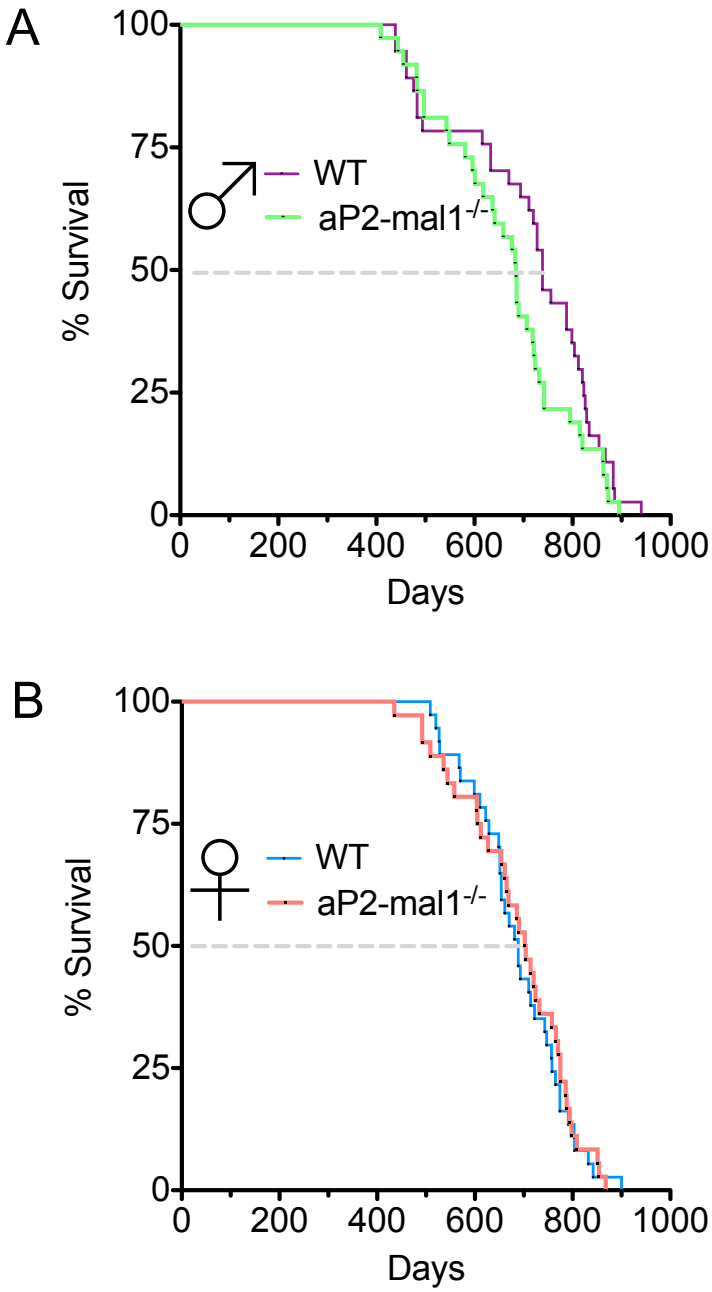

Figure 3-3. Comparison of survival curves for aP2-mal1$^{-/-}$ vs. wild type mice.

Kaplan-Meier curve indicates percent survival for males (A) and females (B) over a period of ~1000 days. N = 40 for each group.

Median survival was 739 and 685 days for wild type and aP2-mal1$^{-/-}$ males, respectively, while female median life spans were 685 and 703 days, respectively (Figure 3-3A and B).

Metabolic fitness in aged mice

Serum lipids are commonly used as indicators of metabolic health. Male FABP deficient animals exhibited numerically higher levels of serum triglycerides, a phenotype that was previously reported in young FABP deficient mice (Cao et al, 2008), yet, this increase did not reach statistical significance for either males or females. Additionally, HDL and VLDL levels were similar between genotypes, in both males and females. LDL content was not determined in two groups (wild type males or FABP deficient females); therefore we were unable to define any similarities or differences for this lipoprotein fraction (Figure 3-4A). Such a profile is seemingly unreflective of an improved metabolic condition, however, we find that FABP deficiency evokes significant qualitative differences in fatty acid species within each lipid class. As such, we previously identified significant elevations in palmitoleate in adult males, which has the potential to mediate specific metabolic effects on liver and muscle to provide major health benefits (Cao et al., 2008).

Prevention of obesity and reduced body weight is another chief indicator of metabolic fitness. Both male and female aP2-mal1$^{-/-}$ mice maintained lower body weights at one year of age compared to their wild type counterparts (Figure 3-4B). Using dual-energy x-ray absorptiometry, we further confirmed that the observed

A

	Total Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
♂ WT	124 ± 7.54	91.2 ± 14.4	93 ± 3.5	ND	18.2 ± 2.85
aP2-mal1$^{-/-}$	127.8 ± 8.749	83 ± 9.4	92.5 ± 6.50	9*	16.8 ± 1.91
♀ WT	106 ± 5.24	61 ± 2.1	84 ± 3.6	7.0 ± 1.7	14.5 ± 2.18
aP2-mal1$^{-/-}$	107 ± 21.5	70.7 ± 3.84	90.3 ± 19.7	ND	14.3 ± 0.882

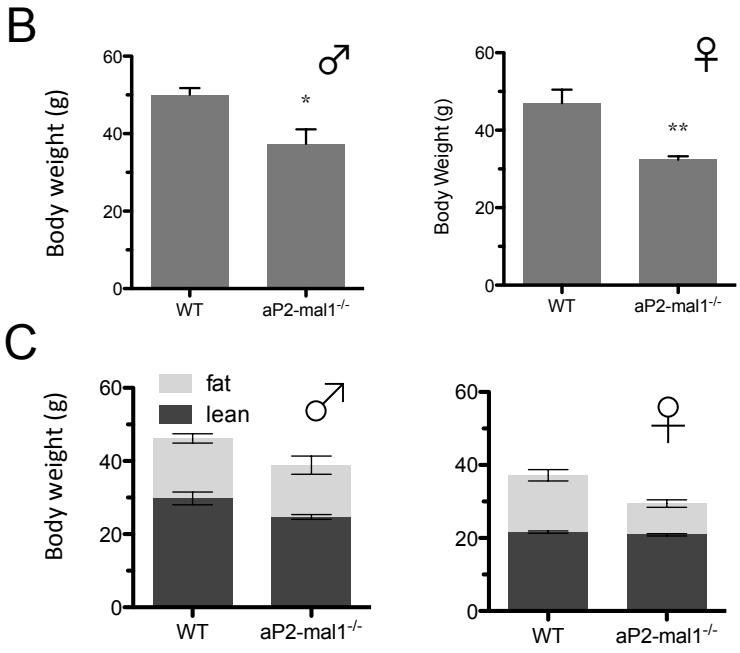

Figure 3-4. Serum lipids and body composition. (A) Circulating levels of cholesterol, TG, HDL, LDL and VLDL. In serum collected after a 6hour daytime food withdrawal (B) Body weight and (C) Body weight composition (lean vs. fat mass). N = 4 for each group. ND = not determined (*) = single animal.

weight reduction was consistent with reduced adiposity and not the loss of lean tissue mass due to muscle wasting (Figure 3-4C).

White adipose tissue

Mounting evidence supports a crucial role for obesity-associated inflammation in the development of insulin resistance and type 2 diabetes (Hotamisligil 2006). Infiltration and activation of adipose tissue macrophages in the obese state produces elevated levels of inflammatory cytokines, which stress the homeostatic mechanisms that maintain glucose levels. We found aP2-mal1$^{-/-}$ deficient adipose tissue in aged males and females to be almost completely void of this characteristic macrophage infiltration (Figure 3-4A,B) and females were profoundly resistant to the presence of inflammatory cytokines (TNFα, IL-6, and IL-10) in circulation (Figure 3-4B).

Liver

FABP deficient animals preserved normal liver appearance, contrasted with signs of steatosis in wild type livers (Figure 3-6A). While neither genotype exhibited particularly high levels of alanine aminotransferase (ALT) in serum, aP2-mal1$^{-/-}$ mice had slightly lower levels than wild type (Figure 3-6B). Hepatic lipid accumulation and elevated ALT levels are routinely used as tools to assess the degree of liver disease. Lack of prominence for either of these elements in aP2-mal1$^{-/-}$ mice marks a trend that correlates with a healthier liver.

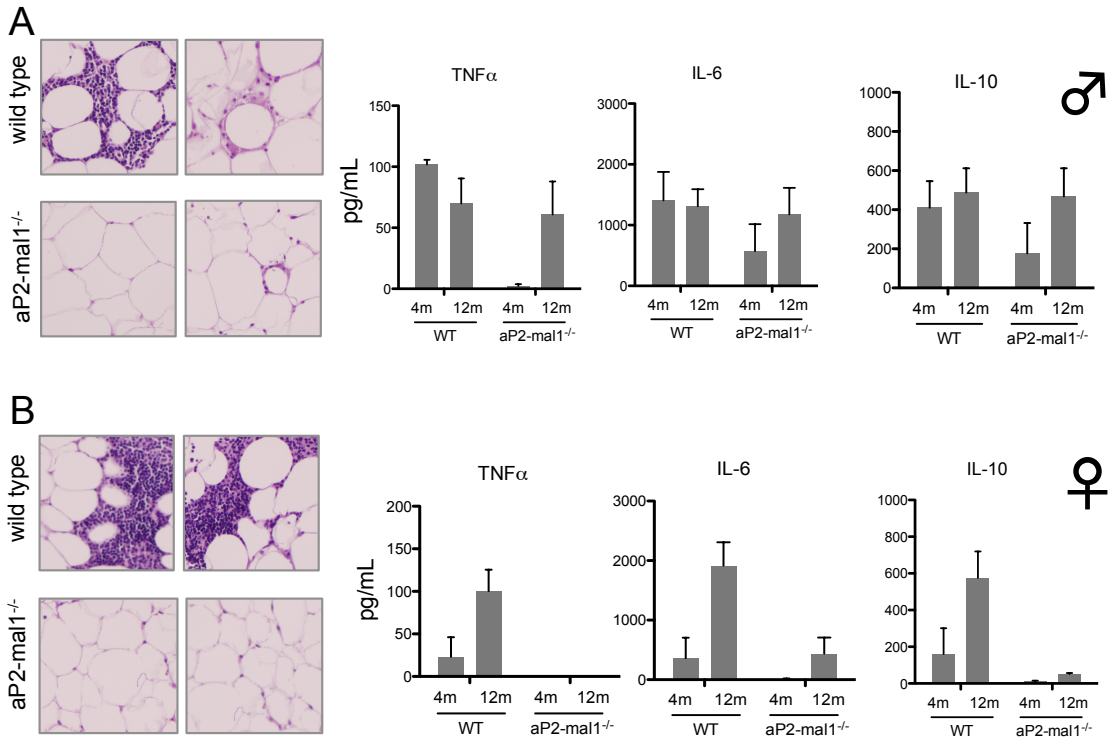

Figure 3-5. White adipose tissue. Hematoxylin and eosin staining of white adipose tissue (representative images from two animals), and circulating levels of inflammatory cytokines in (A) males and (B) females. Cytokine levels in aged mice are compared to levels in young adults.

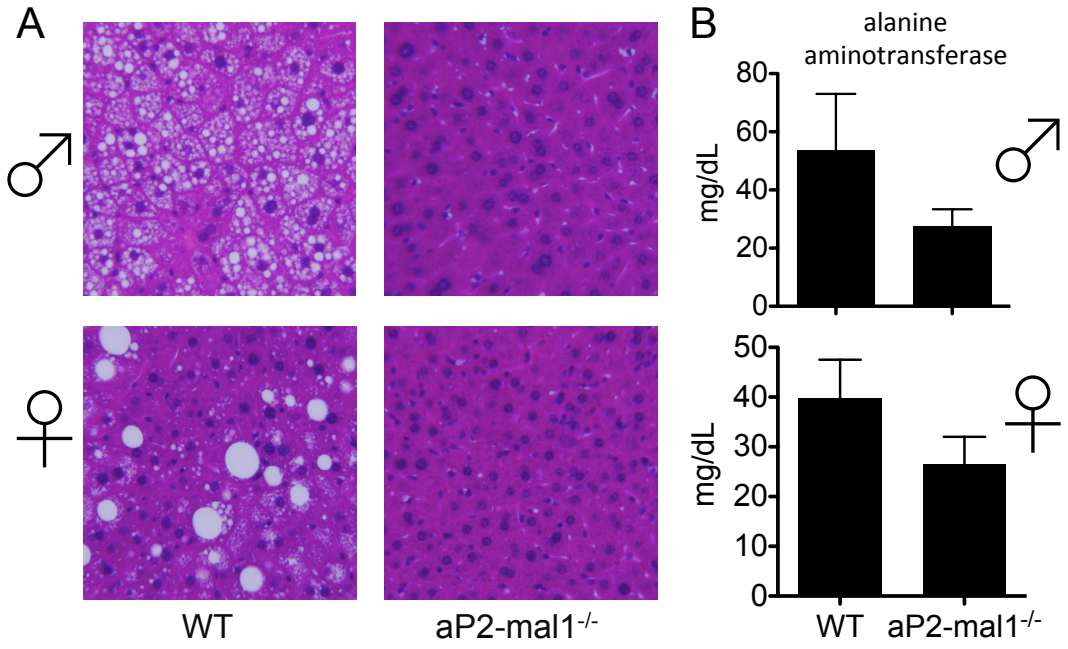

Figure 3-6. Liver tissue. (A) Hematoxylin and eosin staining of liver sections. (B) serum alanine aminotransferase levels.

Glucose homeostasis

Previous data indicated higher glucose tolerance and insulin sensitivity in young adult FABP deficient mice compared to their wild type controls (Maeda et al., 2005). While wild type mice became glucose intolerant and insulin resistant with age, these same parameters were unaffected in FABP deficient animals, in fact the curves are almost identical to published results for young aP2-mal1$^{-/-}$ mice (Figure 3-7A and B) (Maeda et al., 2005). The loss of adiponectin expression is known to promote the development of insulin resistance and glucose intolerance (K. Brochu-Gaudreau et al., 2010). These molecular correlates follow data obtained in female mice, where mean adiponectin levels were 43.4 µg/mL compared to 29.9 µg/mL in wild types (Figure 3-7D). Paradoxically, aged male aP2-mal1$^{-/-}$ mice have considerably less serum adiponectin than their wild type counterparts, a peculiarity also observed in young adult aP2-mal1$^{-/-}$ mice (Maeda et al., 2005). Leptin resistance, marked by a compensatory elevation in serum leptin and de-regulated insulin responsiveness (Flier J. 1997) is particularly clear in aged wild type animals. Serum leptin levels in wild type males and females were 22.9 pg/mL and 25.9 pg/mL, respectively, whereas FABP deficiency permitted the maintenance of normal leptin levels (males, 5.6 pg/mL and females, 3.0 pg/mL) mice (Figure 3-7E). Since the basis for improved insulin sensitivity and glucose tolerance does not depend solely on the action of adipose tissue hormones, we also examined pancreas and related hormonal signals. Pancreas morphology, as illustrated by immunostaining of islets, was comparable between the two genotypes and genders (Figure 3-8A). Relative intensities for β-cell insulin and α-

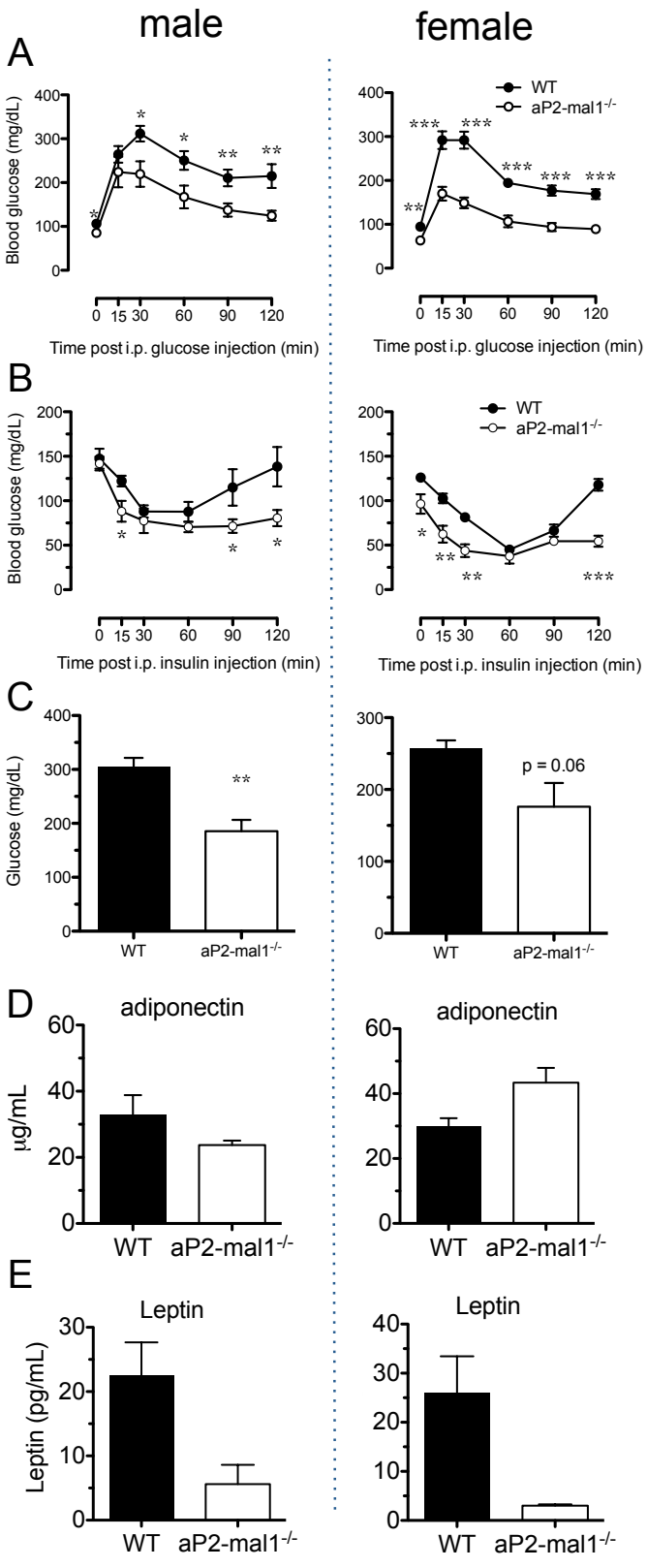

Figure 3-7. Glucose Homeostasis.

Figure 3-7 (Continued). Glucose Homeostasis. (A) Glucose tolerance, (B) insulin tolerance, and serum (C) glucose, (D) adiponectin and (E) leptin levels. Adiponectin and leptin levels in aged mice were compared with measurements in young adults (4 months old).

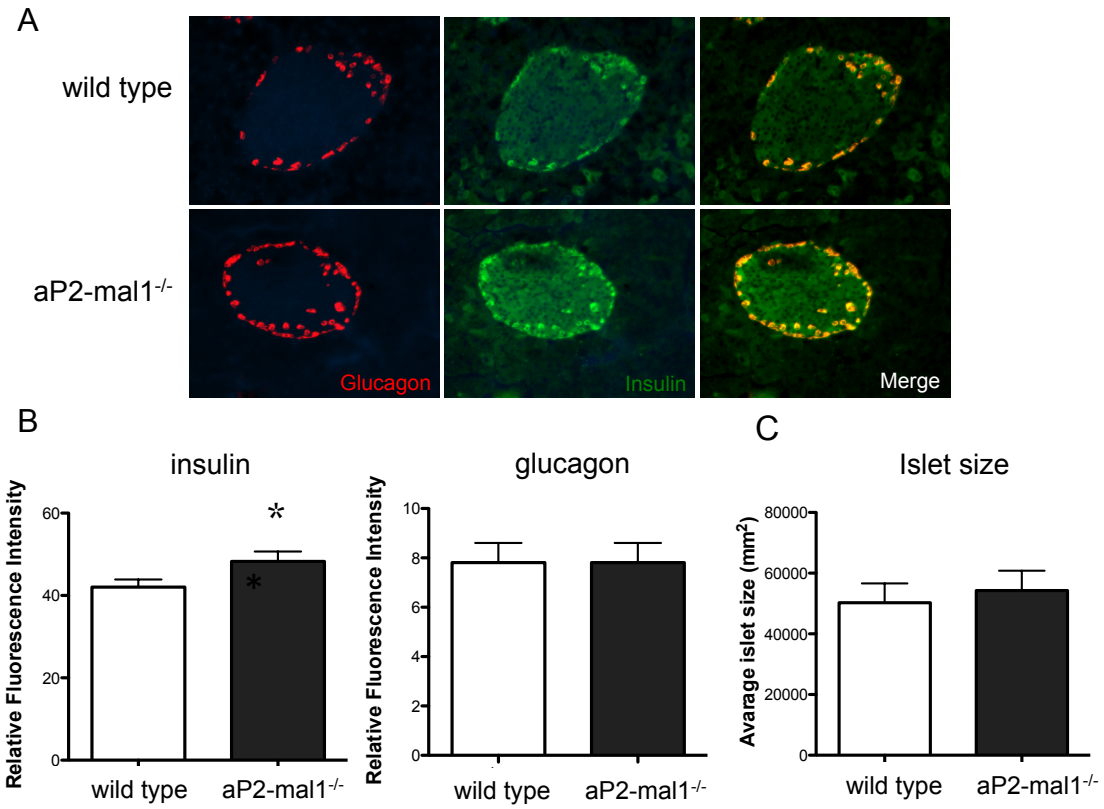

Figure 3-8. Pancreatic islets*. (A) Immunohistochemical staining of glucagon producing β-cells (red) and insulin producing α-cells (green) (yellow = merge). (B) Quantitation of insulin and glucagon fluorescence. (C) Average islet size.

*Combined values for males and females.

cell glucagon were similar across both genders; therefore these values were combined in order to increase statistical power. Relative fluorescent intensities for insulin were higher in FABP deficient mice compared to wild type, indicating that these animals maintain the functional capacity for insulin production.

Physical appearance in aged mice

The dietary restriction field has contributed to a growing body of evidence, which validates the DR intervention as one that actively slows the aging process with respect to organ function, as well as physical appearance (Stern et al., 2001). Thus, in addition to demonstrating the effects of FABP-deficiency on healthspan, we documented the outward phenotype of these animals to determine if these mice also exhibit a delay in visible signs of aging. Both male and female aP2-mal1$^{-/-}$ mice retained a more youthful look. While wild type mice were mangy in appearance, FABP deficient animals retained more of their original coat color and maintained a full coat with intact vibrissae (Figure 3-9).

Metabolic challenge in aged mice

Though we did not reveal any lifespan extending effects of FABP deficiency in our long-term survival study, this determination was made in a cohort of normal fed mice. Hence, it remained a possibility that this genetic intervention possessed limited power to impact survival in normal aging, distinct from the ability to prevent early mortality associated with long-term overnutrition. Therefore, we

challenged a cohort of 40 animals (10 male, 10 female, aP2-mal1$^{-/-}$, 10 male, 10 female, wild type) with high fat diet (HFD). This regimen is known to provoke metabolic stress and further exacerbate insulin resistance. As such, wild type males and females increased blood glucose levels by 75 mg/dL and 101 mg/dL, respectively (Figure 3-10A). In striking contrast, we found that aged aP2-mal1$^{-/-}$ blood glucose levels were virtually unaffected by exposure to HFD, with blood glucose level changing by less than 10 mg/dL in both genders (Figure 3-10A). Surprisingly though, we again detected no difference in survival, despite a profound resistance to one of the major deleterious effects of HFD (Figure 3-10B). Interestingly females of both genotypes were less susceptible to HFD-induced mortality. Mean survival was > 24 months in females, whereas median survival was around 22.6 months in males (Figure 3-10B).

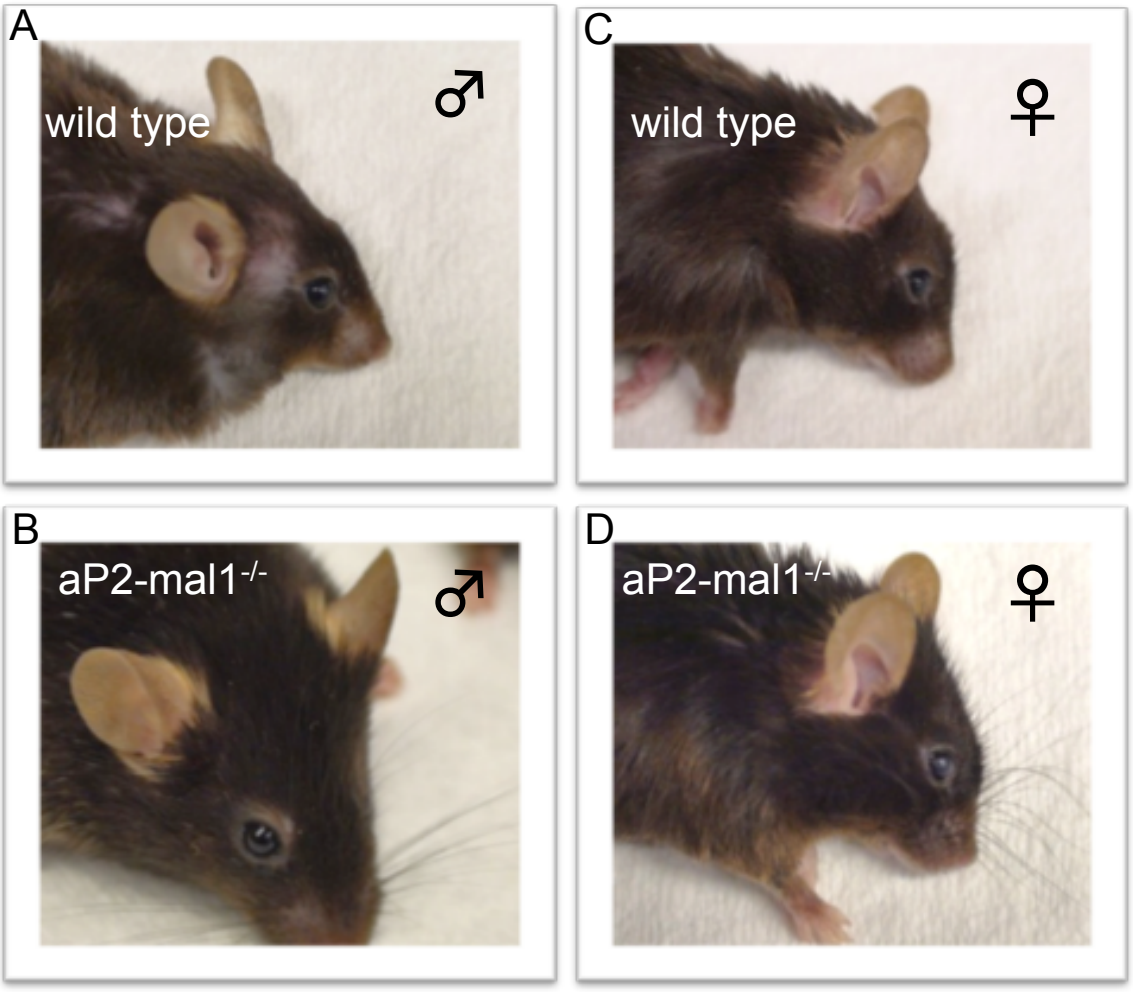

Figure 3-9. Physical appearance of aged mice. Representative photographs of 1-year-old (A) wild type male (B) aP2-mal1$^{-/-}$ male (C) wild type female (D) aP2-mal1$^{-/-}$ female mice.

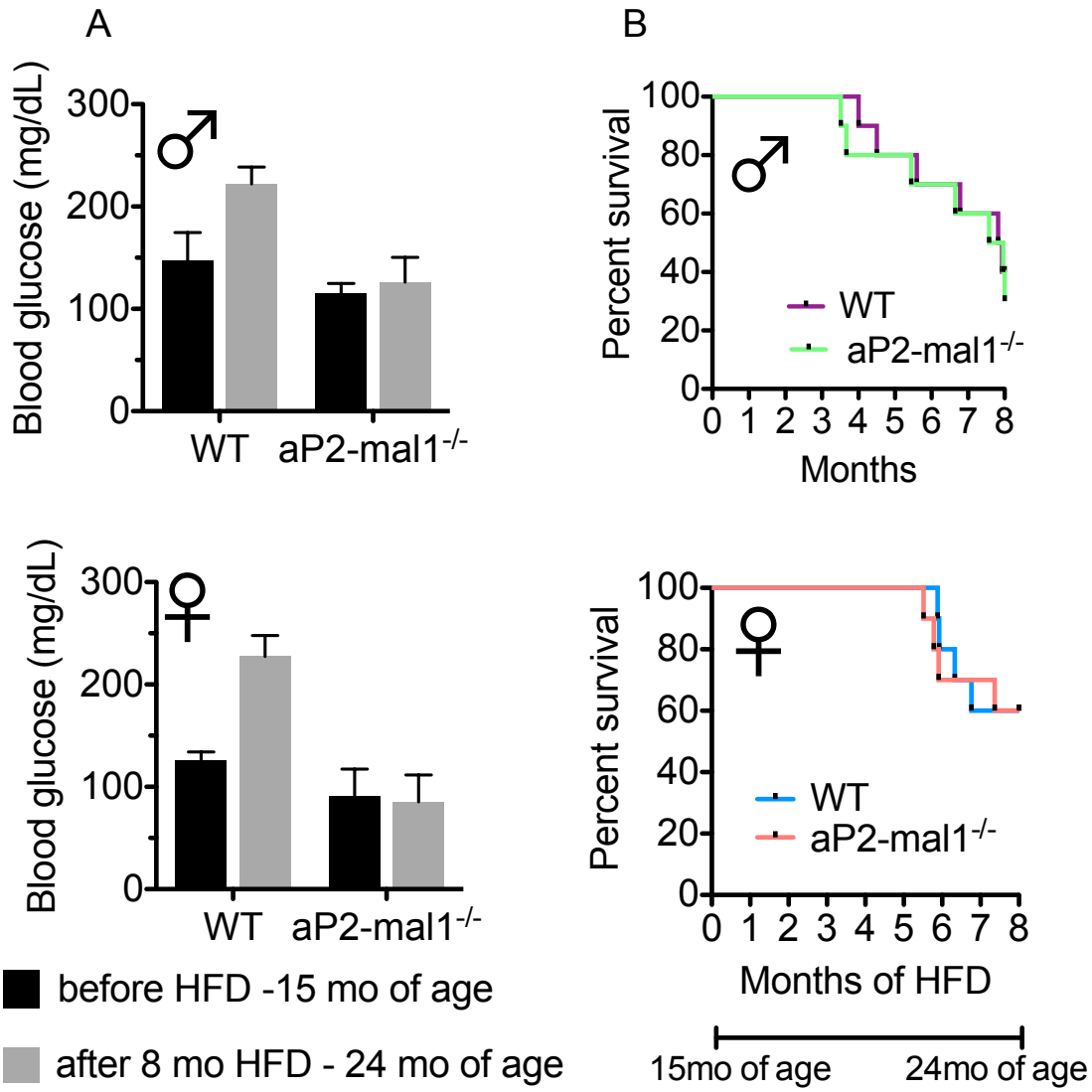

Figure 3-10. Serum glucose and survival after high-fat diet (HFD). At 15 months of age, mice were placed on HFD chow and continued on this diet for 8 months. (A) Blood glucose in males and females after 6hour daytime food withdrawal. (B) Survival after initiation of HFD WT male = 40%; aP2-mal1$^{-/-}$ male = 30%; WT female = 60%; aP2-mal1$^{-/-}$ female = 60%.

DISCUSSION

Steadily, obesity has become more apparent in younger age groups, predisposing them to an early decline in peripheral tissue function, and development of obesity related comorbidities. Perhaps most alarming is that this disproportion may eventually manifest in early mortality for a high number of overweight and obese individuals.

Dietary and genetic methods of increased longevity are known to elicit changes in gene expression that correlate with lower plasma glucose and insulin levels, increased insulin sensitivity, and decreased gluconeogenesis. Given that FABP-deficient mice have been shown to exhibit several of the same health improvements displayed by rodents on restricted diets, we projected that our mice would show signs of increased survival compared to their wild-type counterparts. However, we observed an absence of survival enhancement in FABP deficiency, indicating that the responsible regulatory pathways involved in increasing lifespan are not exclusive to pathways related to improved glucose metabolism and insulin signaling. Identification of common metabolic and transcriptional responses among interventions that increase life span is fundamental to the discovery of genes, metabolites, and pathways underlying mechanisms of aging and for the detection of possible age-related biomarkers. However, despite numerous studies recounting the health benefits of dietary restriction in several species, such a paradigm has provided limited power to detect a mechanism for these effects. In

order to move beyond descriptive phenotypes, evaluation of shared transcriptional and metabolic responses under regimens that enact the same health benefits contributing to longevity could be useful but should be done in parallel with analysis of non-overlapping responses. In light of our results indicating an absence of lifespan extension in FABP deficiency, we thought it would be equally useful to identify transcriptional responses that were unique to the life extending DR model and fail to overlap with phenotypically similar, normal-lived aP2-mal1$^{-/-}$ mice (Table 3-1). In our transcriptional analysis, genes that follow this criterion are secreted peptides, glycoproteins, and proteins involved in redox reactions. This list may contain potential gene families that uphold lifespan extension in a manner that is independent of metabolism and more directly impacts chronological lifespan.

Another quite plausible explanation for lack of lifespan extension is that FABP deficiency promotes deterioration of biological function in a specific pathway or tissue that counteracts the pro-longevity benefit of metabolic fitness. We have no evidence that this is the case for any tissue examined thus far, yet it remains to be thoroughly addressed. In mice, common causes of death are cancer and kidney failure. In our study, gross pathological examination by two separate scientists confirmed that wild type and aP2-mal1$^{-/-}$ mice displayed equal incidences of lymphoma in kidney, liver and spleen, and a comparable degree of glomerulonephritis (data not shown). These findings are also in line with mouse studies using the sirtuin activator—resveratrol, and reports from dietary restriction

studies in primates, where researchers observed robust beneficial health effects and survival that was unchanged from their respective controls (Pearson et al., 2008; Mattison et al., 2012).

Table 3-1. Official gene symbols for significantly altered genes with opposing directionality in aP2-mal1$^{-/-}$ and DR mice

↑AM$^{-/-}$ ↓DR		↓AM$^{-/-}$ ↑DR		
Cidea	Cldn2	Bex2	1700086L19Rik	Spz1
Cox8b	Lcn2	Spink12	Cldn4	Tsga2
Ear1	Slc38a5	Defb15	Tuft1	Znrf4
Chgb	Cyb561	Rpl3l	Calml3	1700016H13Rik
Cd79a	Rbm11	5830403L16Rik	BC025076	5430433G21Rik
Ccl5	Indo	Lcn8	Xkrx	Rhcg
Mmp12	Clu	Cst12	Tmem46	
A130092J06Rik	D730048I06Rik	Lcn12	Defb13	
Orm2	Cryba4	Lcn5	Actg2	
Aard	Spink2	Ntsr1	Acox3	
Sult1a1	Hoxb6	4930525K10Rik	Aass	
Ubd	Slc7a5	Ubqln3	Tnp2	
Hist1h4j	Defb35	4931407G18Rik	Tpd52l1	

While this study indicates that FABPs are perhaps not involved in the adipose tissue response to dietary restriction with respect to increasing lifespan, it does support an underlying role for FABPs in the aging process, with respect to metabolic deterioration. Our study revealed similarities in DR and FABP deficient adipose tissue through a comparative analysis of gene expression and lipid profiles from DR and FABP deficient mice. We discovered a unique overlap in changes to lipid profiles and transcriptional responses when compared to their respective

controls. However, the origin of these molecular links remains to be elucidated. Our comprehensive histological and biochemical examination of metabolic tissues in aged FABP deficient mice illustrated the maintenance of adipose, liver and pancreatic function, and provided further evidence that adipose tissue is a major regulator of the physiological parameters that connect biological outcomes to increased healthspan. Furthermore, this work suggests that the preservation of insulin action and glucose homeostasis alone is not sufficient to extend chronological lifespan, however, it has a significant impact on healthspan. The lack of an identifiable role for FABPs in mouse survival when fed regular diet or after HFD challenge, despite the metabolic similarities with DR, suggests that pathways unrelated to metabolism are involved in the regulation chronological lifespan, and that time of survival is essentially prone to the effects of metabolism on healthspan.

MATERIALS AND METHODS

Comparison of adipose tissue transcriptome and lipidome in dietary-restriction and FABP deficiency

For our large-scale comprehensive analysis of transcriptional regulation in adipose tissue, we used fully annotated and downloadable microarray datasets (Gene Expression Omnibus Set 11845) available from a published study performed in DR mice (Pearson et al., 2008). This study was performed in male C57BL/6NIA mice fed a standard mouse diet *ad libitum* (AIN-93G), or a restricted diet and sacrificed between 18-24 months of age. We used the platform independent software

program HDBStat! (Trivedi et al., 2005) to compare differentially expressed genes between our existing data set for FABP-deficient mice compared to dietary restricted mice in the published data set (ex: DR vs. *ad libitum* and FABP$^{-/-}$ vs. wild type). Raw values were subjected to Z-score transformation to account for differences in signal intensities across platforms. Z-scores greater than 1.5 and less than -1.5 with a p-value threshold of 0.05 and representation in at least 50% of the total arrays were considered significant. These values were generated using the R-statistical package. Since probsets varied between the published array using an Illumina mouseRef-8 v1.1 expression beadchip, and our Affymetrix Mouse Genome 430 2.0 array, we used Resourcerer online database to cross-reference and link the array sources (Tsai et al., 2001). The Database for Annotation, Visualization & Integrated Discovery (DAVID) was used to transform the significantly regulated genes in each data set into functionally related groups (Dennis et al., 2003; Huang et al 2009.)

A similar comparative analysis was performed using the quantitative lipid profiles produced from FABP-deficient vs. WT tissue and dietary restricted vs. *ad libitum* fed tissues. Individual lipid classes from adipose tissue were determined by TrueMass Profiling—TrueMass Lipomic Panel (Lipomics Technologies, West Sacramento, CA). FABP-deficient vs. WT Lipomics data was previously analyzed and reported by Cao et al., 2008 and were utilized in this study for purposes of comparison.

Longevity Experiments

WT and aP2-mal1$^{-/-}$ littermates were generated from heterozygous breeder pairs. Upon weaning, WT and FABP-deficient mice were assigned to a survival cohort and allowed to live out their normal lifespan. Survival was measured as the number of days from birth recorded until death, and analyzed using Kaplan-Meier survival curves. A second cohort of aged mice was used for healthspan studies, gross pathology and microscopic analysis of tissues. These experiments were performed at the Barshop Institute for Longevity and Aging Studies at the University of Texas Health Science Center at San Antonio and the pathology core at Dana Farber Cancer Institute in Boston Massachusetts. All pathological scoring was based on observations from at least two pathologists.

Glucose and insulin tolerance tests

Glucose tolerance tests were performed by *intra peritoneal* injection of glucose at a dose of 1.0g/Kg after a 16hour overnight fast. Insulin tolerance tests were performed by by *intra peritoneal* injection of insulin at a dose of 1U/Kg after a 6hour daytime food withdrawal. Glucose levels were read using Breeze2 glucose test strips.

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CHAPTER 4

NEW PERSPECTIVES AND CONCLUSION POINTS

NEW PERSPECTIVES AND CONCLUSION POINTS

Effects of aP2 inhibition on NAD metabolism may confer stress resistance

Among the theories for how dietary restriction mediates positive effects on aging, is the idea that DR reduces oxidative damage to macromolecules caused by reactive oxygen species (ROS) generated during respiration. Since it has been demonstrated that the metabolic rate in dietary-restricted animals is not altered significantly (McCarter et al., 1985), the reduction in ROS is attributed to an enhanced capacity of DR mice to detoxify ROS, as these mice show increased super oxide dismutase activity in the liver. Although it has not been demonstrated to be the case in mice, over expression of superoxide dismutase alone can extend lifespan in *Drosophila* and yeast (Sun et al., 2004, Harris et al., 2003).

NADH driven oxidative phosphorylation, although beneficial for the cell in terms of ATP production, can become harmful over time. Electrons carried by the electron transport chain are routinely transferred to the terminal electron acceptor, oxygen, ultimately producing water. However, this relatively benign reaction sometimes allows premature electron leakage and the appearance of incompletely reduced intermediate forms of molecular oxygen (O_2^-, H_20_2 and OH) termed reactive oxygen species. Damage to lipids, proteins and DNA can occur when these species react with and disrupt bonds that form these macromolecules (de Magalhaes et al., 2006; Stadtman 2006). Cellular defense mechanisms exist to combat the generation of these harmful species, and their damaging effects. For

example, superoxide dismutases and catalase detoxify superoxides and peroxides, respectively; and thioredoxins can reduce proteins that have been damaged by reacting with ROS. Additionally, excess mitochondrial NADH has been shown to protect against peroxide induced cell death in a human monocytic cell line (U937). By chemically inducing the production of sufficient reducing equivalents to neutralize ROS, authors were able to rescue cells from toxicity (Brambilla et al., 1998).

We tested this paradigm in primary hepatocytes isolated from wild type and aP2-mal1$^{-/-}$ mice by exposure to tert-butylhydroperoxide (tBOOH) and subsequent probing with CellROX Deep Red reagent. We began with primary cells that were equally viable (Figure 4-1), and with increasing concentrations of tBOOH, control hepatocytes exhibited intense fluorescent signal corresponding with an accumulation of ROS (Figure 4-2A). In comparison, FABP cells exhibited low-level diffuse CellROX staining, even at the highest concentration of peroxide, consistent with a markedly reduced presence of ROS (Figure 4-2A). To probe whether or not the inhibition of ROS generation was protective against DNA damage, this same experimental paradigm was used. For this, we employed single cell gel electrophoresis of oxidant exposed primary hepatocytes and subsequent staining with SYBR green to detect strand breaks. Three-dimensional surface plots illustrate "comets" that are representative of those generated under the highest concentration of tBOOH (Figure 4-2B). Higher concentrations of tBOOH (4.8mM)

were required to induce DNA damage than that which induced ROS (2.4mM). Despite this, wild type hepatocytes were still more sensitive to oxidant exposure and produced comet tails of greater length and higher density, corresponding to a modest increase in levels of DNA damage, compared to aP2-mal1$^{-/-}$ cells (Figure 4-2C-D). It is possible that the decreased presence of tBOOH induced DNA damage in hepatocytes was related to the elevated expression of liver PARP (as demonstrated in Figure 2-3A and 2-3B). Taken together, these data support an antioxidant role for surplus NADH and designates fatty acid binding proteins as molecules that can potentially counteract the restorative nature of liver.

In this setting, we can follow-up this finding by determining if NAD metabolism is altered in other peripheral tissues, if additional cell types exhibit cytoprotection, and/or whether these cell types are protected against additional physiological stressors. Correlating these outcomes with FABP-deficiency will strengthen our hypotheses regarding the role of aP2 in counteracting the recuperative ability of metabolic tissues.

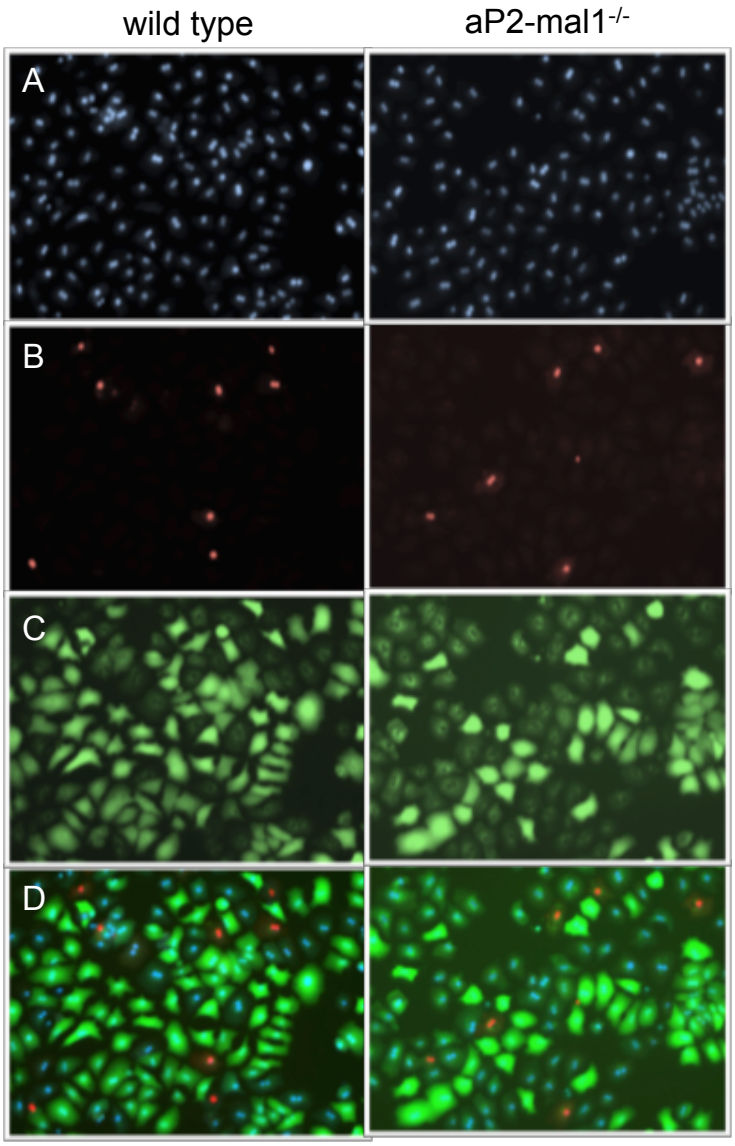

Figure 4-1. Cell Viability of primary hepatocytes after isolation

(A) DAPI; nuclei (B) ethidium homodimer-1; dead cells (C) calcein-AM; live cells (D) overlay of A-C.

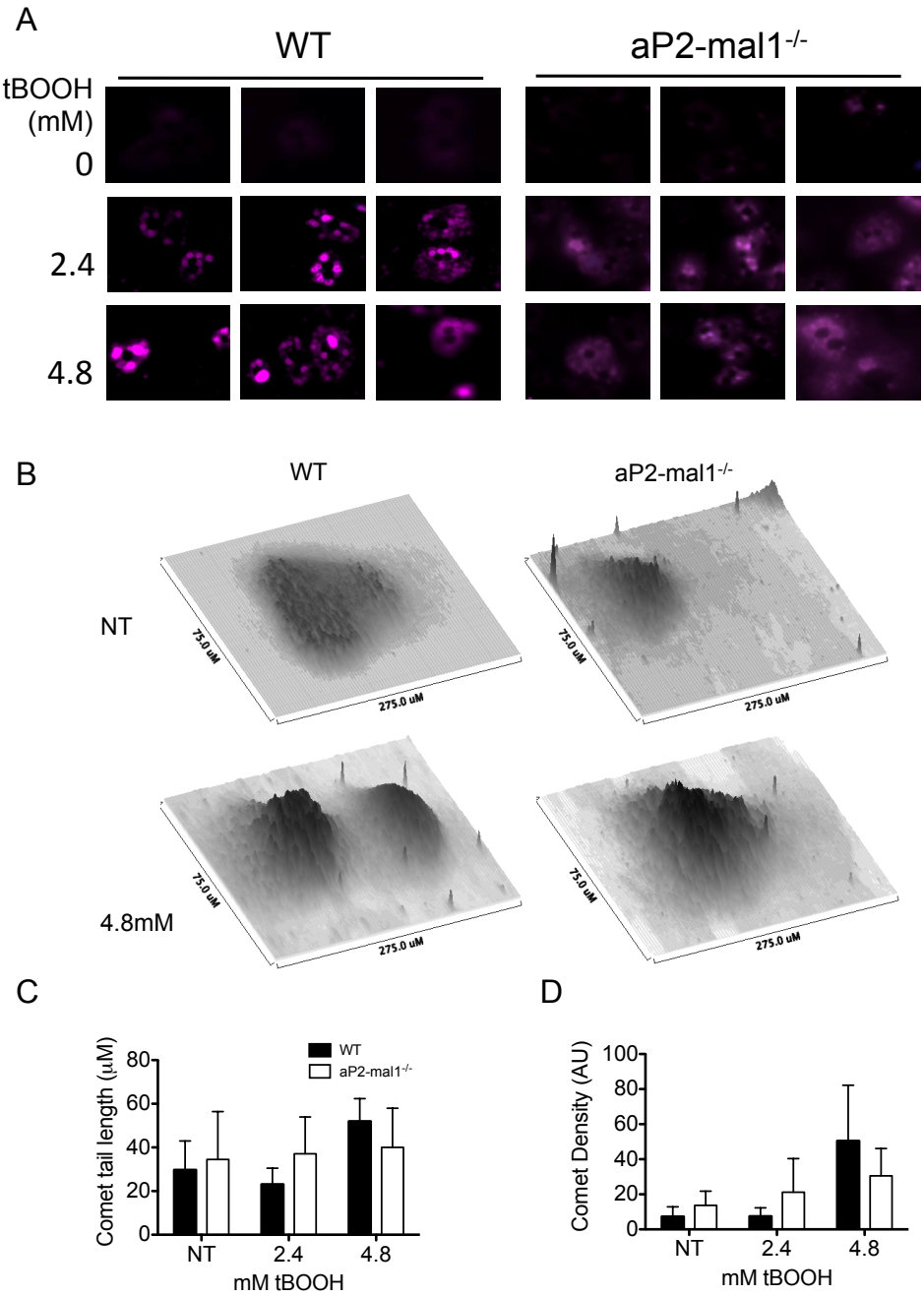

Figure 4-2. Cytoprotection in aP2-mal1$^{-/-}$ primary hepatocytes.

Figure 4-2 (Continued). Cytoprotection in aP2-mal1$^{-/-}$ primary hepatocytes.

(A) *Protection from ROS species generation*. Primary hepatocytes were incubated for 30min with indicated concentrations of tBOOH. ROS species detected with CellROX Deep Red reagent (B-C) *Comet tail length* (B) and *density* (C). Primary hepatocytes were exposed to tBOOH as in panel A. Quantitation of comet properties, including average tail length and tail density were determined using Image J. (E). Surface Plots. Representative 3-D surface plots of SYBR green fluorescent images. Top panels, no treatment (NT) bottom panels, 4.8mM tBOOH.

aP2 as a potential therapeutic target and biomarker for metabolic aging

Chapter 2 describes a role for aP2 in directing substrate utilization and NAD metabolism in hepatocytes (Figure 4-3). The implications of these effects are far reaching, given that NAD is central to many fundamental metabolic processes. The energy stored in reduced NAD supplies metabolic flux in all living cells, without which, many biochemical reactions would be rendered useless. Regulation of NAD metabolism is critical for processes that convert nutritional energy to cellular energy. Thus, the modulation of NAD levels by targeting aP2 carries with it, the potential to reprogram these metabolic events. Furthermore, alterations in adipose tissue lipidomic and transcriptional profiles identified in Chapter 3 can potentially have profound effects on levels of secreted factors present in serum, which contribute to systemic metabolism and extension of healthspan.

Criteria for biomarkers of aging set by Redman (Redman et al., 2008) put forth that the proposed factor must reflect physiological or functional age as demonstrated by temporal changes in baseline levels of a given factor, such as an expressed gene or molecule. Biological response to this factor must be slowed or reversed by caloric restriction and perhaps most crucial, the marker must be reliably measured. To date, the most reliable measures in mammals that meet these criteria are circulating levels of hormones (i.e. insulin, IGF) (Redman et al., 2008). Although FABP-deficient mice have significantly lower circulating levels of insulin compared to normal mice when both are placed on a high-fat diet, on

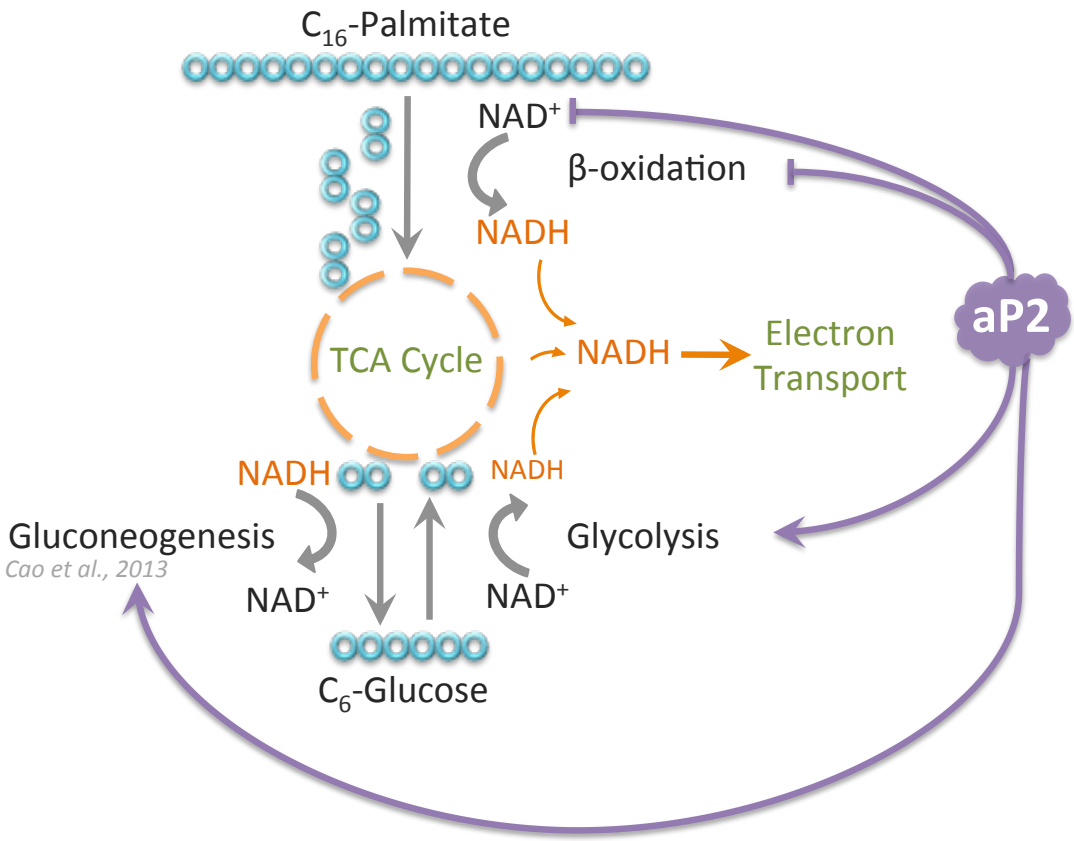

Figure 4-3. Regulatory model for adipose tissue fatty acid binding protein 4 (aP2). Role for aP2 in directing substrate usage through the regulation of NAD, glucose and fatty acid metabolism.

regular diet, insulin levels are comparable, at least as determined up to 9-months of age (Maeda et al., 2005). Therefore, in some settings, insulin does not serve as a reliable biomarker for metabolic aging. Though it is known that serum aP2 levels increase with adiposity, and that adiposity increases with age, it would be useful to measure serum levels of aP2 in wild type animals over time in order to determine if there are temporal changes that occur during the aging process. If so, aP2 may be employed as a biomarker that is consistent with biological aging.

Though we have now identified several protective features of FABP deficiency in the context of obesity and aging, it is important to thoroughly evaluate the effects of this intervention on adipocyte health and whole body organ function. Much like the design of simple machines such as levers or pulleys, drug therapies make synthetic connections between the existing input and the desired outcome; and like simple machines, potential therapeutics must be evaluated for their efficiency. It is important to identify a possible trade-off when reprogramming metabolism through inhibition of aP2. And if so, is the trade-off significant? Though this study did not identify any deleterious effects of acute or long-term adipose tissue FABP deficiency, there are many functional aspects of this protein that remain unknown. This current work advances our understanding of aP2 mediated metabolic functions, however, more work is also necessary to further our understanding of factors that alter the functional characteristics of aP2. Several potential sites for modification exist within adipose tissue, circulation, and in the

periphery, where aP2 may encounter post-translational modifications or specific lipid ligands. Nevertheless, our studies support the possibility for pharmacologic manipulation of FABPs as a potential approach to combat the effects of metabolic disease and aging.

MATERIALS AND METHODS

Reactive oxygen species and DNA damage detection

Primary hepatocytes were seeded onto glass coverslips and allowed to recover for 24h after isolation. Cells were washed once with PBS and incubated with indicated concentrations of tert-butylhydroperoxide for 30 min at 37^0C, 5% CO_2. The cells were washed once with PBS allowed to recover from oxidant exposure for 4h in DMEM containing 10% calf serum and used immediately for detection of reactive oxygen species or DNA damage. For ROS detection, cells were incubated with Cell ROX deep red reagent at a dilution of 1:500 (Invitrogen #C10422) for 30 minutes, washed once with PBS and fixed for 15min using 3.7% formaldehyde solution. Fluorescence was measured using the far-red filter on a Leica microscope. To detect DNA damage, a CometAssay reagent kit (Trevigen # 4250-050-K) was used. Cell pellets were collected into the provided alkaline buffer and electrophoresed under alkaline conditions for the detection of both single stranded and double stranded breaks. Image J was used for quantitation of comet tail length and

density, N >50 for each condition. Cell viability after each isolation was determined using the LiveDead Assay (Invitrogen).

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