IDENTIFICATION AND CHARACTERIZATION OF ALTERED

MITOCHONDRIAL PROTEIN ACETYLATION IN FRIEDREICH'S ATAXIA

CARDIOMYOPATHY

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ABSTRACT

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Friedreich's Ataxia (FRDA) is a rare and poorly understood autosomal
recessive disease caused by a pathological deficiency of the mitochondrial
protein frataxin. Patients suffer neurodegeneration, ataxia, diabetes, and heart
failure. In an effort to understand the mechanisms of heart failure in FRDA, we
investigated the role of the protein modification acetylation, which is highly
abundant on mitochondrial proteins and has been implicated in regulating
intermediary metabolism. Using mouse models of FRDA, we found that cardiac
frataxin deficiency causes progressive hyperacetylation of mitochondrial proteins
which is correlated with loss of respiratory chain subunits and an altered
mitochondrial redox state. Mitochondrial protein hyperacetylation could be
reversed by the mitochondria-localized deacetylase SIRT3 *in vitro*, suggesting a
defect in endogenous SIRT3 activity. Consistently, frataxin-deficient cardiac
mitochondria showed significantly decreased rates of fatty acid oxidation and
complete oxidation to carbon dioxide. However, the degree of protein
hyperacetylation in FRDA could not be fully explained by SIRT3 loss. Our data
suggested that intermediary metabolites and perhaps acetyl-CoA, which is

required for protein acetylation, are accumulating in frataxin-deficient mitochondria. Upon testing the hypothesis that mitochondrial protein acetylation is non-enzymatic, we found that the minimal chemical conditions of the mitochondrial matrix are sufficient to cause widespread non-enzymatic protein acetylation *in vitro.* These data suggest that mitochondrial protein hyperacetylation in FRDA cardiomyopathy mediates progressive post-translational suppression of mitochondrial oxidative pathways which is caused by a combination of SIRT3 deficiency and, likely, an accumulation of unoxidized acetyl-CoA capable of initiating non-enzymatic protein acetylation. These findings provide novel insight into the mechanisms underlying a poorly understood and fatal cardiomyopathy and highlight a fundamental biochemical mechanism that had been previously overlooked in biological systems.

R. Mark Payne, M.D., Chair

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LIST OF ABBREVIATIONS

AceCS2	acetyl-CoA synthetase 2
AdPEO	autosomal dominant progressive external ophthalmoplegia
ANT1	adenine nucleotide translocase
BSA	bovine serum albumin
COI	cytochrome oxidase I
CO_2	carbon dioxide
FRDA	Friedreich's Ataxia
GNAT	Gcn-5-related N-acetyltransferase
GST	glutathione S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
ISC	iron-sulfur cluster
ISCU	iron sulfur scaffold unit
KAT	lysine acetyltransferase
KDAC	lysine deacetylase
LCAD	long chain acyl-CoA dehydrogenase
LHON	Leber's Hereditary Optic Neuropathy
MCAD	medium chain acyl-CoA dehydrogenase
MCK	muscle-creatine kinase
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide

NAM	nicotinamide
NDUFA9	NADH dehydrogenase (ubiquinone) alpha subcomplex 9
NSE	neuron-specific enolase
PAGE	polyacrylamide gel electrophoresis
PPARα	peroxisome proliferator activated receptor alpha
PPARγ	peroxisome proliferator activated receptor gamma
PTM	post-translational modification
ROS	reactive oxygen species
SDHB	succinate dehydrogenase iron-sulfur subunit
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid
VDAC	voltage-dependent anion channel
WCL	whole cell lysate
4-HNE	4-hydroxy-2-nonenal

CHAPTER I: INTRODUCTION

The genetic and molecular basis of Friedreich's Ataxia

In 1863, a disease characterized by progressive neurodegeneration and loss of motor coordination and walking ability (ataxia) was observed in five patients by the German professor of pathology, Nicolaus Friedreich, and soon became known as Friedreich's Ataxia (FRDA) (*1*). In 1996, an international team led by Professors Massimo Pandolfo and Michel Koenig identified the causal genetic defect in Friedreich's Ataxia patients (*2*). Their earlier linkage analysis using Friedreich's Ataxia patient samples had mapped the segregating disease region to a 150 kilobase fragment located on chromosome 9q13-q21.1 (*3*). They then performed an EcoR1 restriction digest mapping of the critical region and found that a fragment corresponding to intron 1 of the *X25* gene was, on average, 2.5 kb larger in FRDA patients and carriers than in controls. After sequencing this fragment, they discovered a GAA·TTC triplet nucleotide repeat expansion in 98% of FRDA patients and carriers that ranged in size from approximately 200 to 900 repeats, while control samples contained 7 to 32 repeats. The remaining two percent of patients were compound heterozygous for the GAA expansion and a point mutation in the *X25* coding region. Thus, the international research team found that Friedreich's Ataxia was an autosomal recessive disorder caused by a triplet nucleotide repeat expansion located in intron 1 of the *X25* gene (*2*). This gene is now known as *FXN* and its protein product is known as Frataxin.

Given that the FRDA patients identified as compound heterozygous harbored putative loss-of-function mutations in the *FXN* gene, it was hypothesized that the other GAA-repeat containing allele generated less gene product through defects in either messenger RNA (mRNA) splicing or gene transcription. Indeed, studies soon confirmed that the GAA-repeat expansions in the *FXN* gene were associated with minimal to undetectable levels of Frataxin mRNA in FRDA patient tissues (*2*). Bidichandani, et al. determined that Frataxin mRNA deficiency in FRDA patients was unrelated to a splicing defect and, instead, was inversely related to the size of the GAA repeat expansions; the longer the GAA repeat, the less Frataxin mRNA generated in an *in vitro* transcription assay (*4*). The authors proposed several models through which the intronic GAA repeat tract could facilitate the formation of non-Watson-Crick base pairing DNA triplex structures, which could then interfere with *FXN* transcription. Additional work extended this study by demonstrating that the GAA repeats do not interfere with transcription initiation, rather they impede transcriptional elongation. The B-triplex DNA structures likely inhibit the extension of RNA polymerase, resulting in less Frataxin mRNA transcript and, consequently, less Frataxin protein product (*5*).

Although an expanded GAA repeat in intron 1 of the FXN gene is the primary cause of Friedreich's Ataxia, there is substantial evidence implicating epigenetic silencing of the *FXN* locus as a secondary cause underlying impaired transcription of Frataxin mRNA. Saveliev, et al. found that short repeat expansions found in myotonic dystrophy and FRDA promote the local formation

of heterochromatin, reduce promoter accessibility, reduce transcription of engineered transgenes, and recruit heterochromatin protein 1 (HP1) (*6*). These observations indicated that the GAA expansions in FRDA likely promote transcriptional silencing of the *FXN* locus in a manner akin to position effect variegation where genes adjacent to heterochromatic regions become silenced. In support of this notion, Herman et al. demonstrated that the *FXN* locus in FRDA lymphoblast cell lines exhibits hypoacetylation on Histones 3 and 4, consistent with the formation of heterochromatin and transcriptional repression (*7*). A significant increase in methylation of the Frataxin promoter has also been observed in a large FRDA patient cohort. Furthermore, increased methylation was inversely correlated with Frataxin mRNA expression (*8*). Taken together, these studies indicate that triplet repeat-induced transcriptional silencing, in addition to transcriptional blockade induced by aberrant DNA structures, contributes to impaired transcription of the *FXN* gene.

The *FXN* gene and the function of its gene product, Frataxin

In humans, the *FXN* gene consists of 7 exons spanning an 80 kilobase region located on chromosome 9q21.11 (*9*). The *FXN* gene/protein is highly conserved throughout evolution, having homologs in chimpanzee (*Pan troglodytes*) to single-celled eukaryotes such as yeast (*Schizosaccharomyces pombe*) (Figure 1.1). Human *FXN* produces three transcript variants according to the most recent entry in the National Center for Biotechnology Information (NCBI) Database. The longer transcript variant (NM_000144.4) represents the canonical

transcript while the other variants may be artifacts and currently have unknown

function. This translated transcript produces a 210 amino acid protein known as

Frataxin.

```
1    MWTLGRRAVA-GLLASP-SPAQAQTLTRVPRPAELAPLCGRRGLRTDIDATCTPRRASSN    58   Q16595   FRDA_HUMAN
1    MWAFGGRAAV-GLLPRTASRA--SAWVGNPRWREPIVTCGRRGLHVTVNAGATRH-AHLN    56   O35943   FRDA_MOUSE
1    --MFAGRLMVRSIVGRA-------CLATMGRWSKPQAHASQVILP------STPAIAAV-  44   Q9W385   FRDA_DROME
1    MWTLGRRSVA-SFLPRSALPGFAPTRAGAPRPAKDLSLSGLPGLRIGTAKAPARSQSSLS   59   Q05B87   FRDA_BOVIN
1    --------------------------------------------------------MLS-  3   Q9TY03   FRDA_CAEEL
1    ---MIKRSLA-SLV---------------RV---SSVMGRRYMIAA--AGGERARF---  32   Q07540   FRDA_YEAST


59   QRGLNQIWNVKKQSVYLMNLRKS-----GTLGHP--GSLDETTYERLAEETLDSLAEFFE   111   Q16595   FRDA_HUMAN
57   -LHYLQILNIKKQSVCVVHLRNL-----GTLDNP--SSLDETAYERLAEETLDSLAEFFE   108   O35943   FRDA_MOUSE
45   --------AIQCEEFTANRRLFS-----SQIETE--STLDGATYERVCSDTLDALCDYFE    89   Q9W385   FRDA_DROME
60   LRCLNQTLDVKKQSVCWINLRTA-----GTLGDA--GTLDDTTYERLAEETLDSLAEFFE   112   Q05B87   FRDA_BOVIN
4    --------TILRN--NFVRRSFS-----SR-------IFSQNEYETAADSTLERLSDYFD    41   Q9TY03   FRDA_CAEEL
33   ---CPAVTNKKNHTVNTFQKRFVESSTDGQVVPQEVLNLPLEKYHEEADDYLDHLLDSLE    89   Q07540   FRDA_YEAST
              .         .         .          :     *.  ...  *:  *  :  ::

112  DLADKPYTFEDYDVSFGSGVLTVKLGGDLGTYVINKQTPNKQIWLSSPSSGPKRYDWTG-   170   Q16595   FRDA_HUMAN
109  DLADKPYTLEDYDVSFGDGVLTIKLGGDLGTYVINKQTPNKQIWLSSPSSGPKRYDWTG-   167   O35943   FRDA_MOUSE
90   ELTENASELQGTDVAYSDGVLTVNLGGQHGTYVINRQTPNKQIWLSSPTSGPKRYDFVGT   149   Q9W385   FRDA_DROME
113  DLADKPYTFEDYDVSFGSGVLTVKLGGDLGTYVINKQTPNKQIWLSSPSSGPKRYDWTG-   171   Q05B87   FRDA_BOVIN
42   QIADSFPVSEQFDVSHAMGVLTVNVSKSVGTYVINKQSPNKQIWLSSPMSGPKRYDLE--    99   Q9TY03   FRDA_CAEEL
90   ELSEAHPDC-IPDVELSHGVMTLEIP-AFGTYVINKQPPNKQIWLASPLSGPNRFDLLN-   146   Q07540   FRDA_YEAST
     ::::           **   . **:*:::     ******:* *******:**  ***:*:*

171  ---KNWVYSHDGVSLHELLAAELTKALKT-KLDLSSLAYSGKDA------   210   Q16595   FRDA_HUMAN
168  ---KNWVYSHDGVSLHELLARELTKALNT-KLDLSSLAYSGKGT------   207   O35943   FRDA_MOUSE
150  VAAGRWIYKHSGQSLHELLQQEIPGILKSQSVDFLRLPYCS---------   190   Q9W385   FRDA_DROME
172  ---RNWVYSHDGVSLHELLATELTQALKT-KLDLSALAYSGKDTCCPAQC   217   Q05B87   FRDA_BOVIN
100  -EEGKWTYAHDGEQLDSLLNREFRKILADDRIDFSRHV------------   136   Q9TY03   FRDA_CAEEL
147  ---GEWVSLRNGTKLTDILTEEVEKAISKSQ------------------   174   Q07540   FRDA_YEAST
         .*    :.*  .*  .:*   *.    :
```

Figure 1.1 Frataxin protein sequence aligned and conservation. Uniprot BLAST protein sequence alignment for Frataxin across diverse species indicates strong evolutionary conservation. DROME, *D. Melanogaster* (Fruit Fly); BOVIN, Cow; CAEEL, *C. elegans* (roundworm).

Human Frataxin is translated as a precursor polypeptide containing an N-

terminal mitochondrial targeting sequence spanning amino acids 1-41. This

Frataxin precursor is trafficked to the mitochondrion and imported into the

mitochondrial matrix via the import pore machinery and then processed into at

least two different forms by the mitochondrial processing peptidases (MPPs) (*10,

11*). The first processing cleavage removes the 41 amino acid mitochondrial

targeting sequence to generate Frataxin[42-210]. This intermediate form is then

further processed, removing N-terminal amino acids 42-80 to yield the mature

protein composed of amino acids 81-210, which resides in the mitochondrial matrix. Frataxin is transcribed and expressed in every type of mammalian tissue, but it is particularly abundant in the central nervous system, dorsal root ganglia, and heart (*12*).

The exact biochemical function of Frataxin remains undefined. However, there is a consensus in the literature that Frataxin has a role in several aspects of mitochondrial iron homeostasis. The first functional studies in yeast indicated that deletion of the yeast Frataxin homolog resulted in reduced oxygen consumption and impaired mitochondrial oxidative phosphorylation (*13*). Furthermore, overexpression of this Frataxin homolog in a yeast strain unable to grow on iron-deficient medium partially alleviated the growth defect, suggesting Frataxin functioned in iron mobilization (*13*). Systemic ablation of Frataxin in mice is incompatible with embryonic development, highlighting an essential function for Frataxin in mammalian systems (*14*). Conditional ablation of Frataxin in the heart and brain (Neuron Specific Enolase-Cre, NSE-Cre), or heart and skeletal muscle (Muscle Creatine Kinase-Cre, MCK-Cre) of mice causes degeneration of the dorsal root ganglia, ataxia, and deficiency of respiratory complexes I, II, and III, and aconitase in the heart and brain stem (*15*). Furthermore, there is a late onset accumulation of iron deposits in the myocardium in the longer-lived MCK-Cre animals (*15*). Frataxin is known to act as a citrate-dependent iron chaperone responsible for modulating the assembly of the labile 4Fe-4S iron-sulfur cluster of mitochondrial aconitase (*16*). Additionally, Frataxin interacts with multiple members of the mitochondrial iron-sulfur cluster assembly machinery including

the cysteine desulfurase also known as NFS1, the iron-sulfur cluster scaffold unit (ISCU) and ISD11, forming a 190 kDa complex (*17*). There is also evidence suggesting that the Frataxin[42-210] intermediate oligomerizes to sequester intra-mitochondrial iron (*11*). Together, these studies indicate that Frataxin has an essential role in the metabolism of iron, particularly in the proper assembly and function of intra-mitochondrial and, perhaps, extra-mitochondrial iron-sulfur cluster dependent proteins.

The biochemical and molecular consequences of Frataxin deficiency

With knowledge gained from human patients as well as animal and cellular models, recent investigations have uncovered a great deal of information toward a better understanding of the disease mechanisms underlying FRDA pathogenesis. Although the true function of Frataxin remains undefined, its best understood role is in the biosynthesis and function of mitochondrial iron-sulfur cluster-dependent proteins. The earliest functional studies of Frataxin deficiency following the identification of the causal gene defect demonstrated impaired activity of the iron-sulfur cluster proteins of the Electron Transport Chain (ETC) including Complex I, II, and III (*18*). This study also discovered that mitochondrial aconitase of the tricarboxylic acid (TCA) cycle also displayed impaired activity. This seminal work revealed FRDA as a mitochondrial disorder and provided the basis for understanding Frataxin's role in mitochondrial iron homeostasis. In addition to its role in ISC assembly, Frataxin was shown to play an active role as a citrate-dependent iron chaperone involved in aconitase activation (*16*).

Consistent with impaired ETC activity in FRDA, phosphorus magnetic resonance spectroscopy studies indicated reduced ATP production in patient skeletal muscle and heart (*19, 20*). Furthermore, the level of energy deficit in the patients studied was strongly correlated with the degree of cardiac hypertrophy, highlighting the importance of impaired energy homeostasis in FRDA cardiomyopathy. The majority of the mitochondrial and biochemical defects identified in human patients have also been recapitulated in mouse models of FRDA (*15, 21*), which have provided valuable systems for testing potential therapeutic interventions (*22, 23*).

Although ISC enzyme deficiency and consequently impaired energy generation is widely regarded as the major pathogenic mechanism underlying FRDA, there are also important arguments for disrupted mitochondrial and cellular iron homeostasis as late-onset factors of disease progression. Iron deposition in cardiomyocytes often accompanies myocardial hypertrophy in FRDA (*24*), suggesting a role for iron toxicity-mediated oxidative tissue damage. However, the myocardial iron-positive granules only become evident upon post-mortem tissue analysis which limits an accurate interpretation of a role for iron dyshomeostasis in disease progression. A detailed analysis of the Neuron-specific enolase (NSE) and Muscle Creatine Kinase (MCK) mouse models of FRDA demonstrated that cardiac hypertrophy and mitochondrial iron-sulfur cluster protein defects precede any evidence of myocardial iron-deposition, suggesting that iron accumulation may be a secondary disease phenotype (*15*). Nonetheless, the MCK mouse models do eventually develop mitochondrial iron

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deposition along with marked rearrangement of genes involved in mitochondrial and cellular iron import and storage (*25*). Attempting to limit cardiac iron deposition in the MCK mouse models via a mitochondrial permeable iron chelator limited cardiac iron-loading and hypertrophy (*22*). Another study of iron chelation therapy in a Frataxin-knockdown cell line demonstrated an increase in aconitase activity as well as improvements in mitochondrial membrane potential and ATP production (*26*). Together, these studies indicate that a dysregulation of mitochondrial iron metabolism may ultimately be important in the progression of FRDA.

An increased sensitivity to oxidative stress is also likely to contribute to disease progression in FRDA. Reactive oxygen species (ROS) are a physiological byproduct of aerobic mitochondrial respiration that, in excessive quantities, can cause damage to cellular macromolecules including DNA, protein, and lipid membranes and may ultimately lead to apoptotic cell death. Although healthy cells have robust mechanisms for mitigating ROS, evidence indicates that Frataxin-deficient cells are less capable of coping with oxidative insults (*27, 28*). This increased sensitivity or lower threshold for oxidative stress may be causally related to disrupted iron utilization, but may also represent an independent factor in the disease development. Early work demonstrated that FRDA patient fibroblasts are hypersensitive to hydrogen peroxide and that this sensitivity is partially rescued through iron chelation (*27*). An additional investigation also noted that mitochondrial manganese superoxide dismutase (MnSOD) failed to be induced in both FRDA patient fibroblasts and in the heart of

mouse models (*28*). The increased sensitivity to ROS in FRDA has also been supported through studies in yeast and human lymphoblasts indicating impairments of the glutathione defense to ROS (*29*). Although there may be an increased vulnerability to oxidative damage due to impaired endogenous antioxidant defenses, research in animal models suggests that oxidative stress, by itself, is not a notable effect of Frataxin deficiency (*30*). Thus, it is important to distinguish between increased basal levels of oxidative stress and an increased sensitivity to oxidative stress, as this has been a topic of considerable controversy in FRDA research (*30*). Nevertheless, the continuous bioenergetic and mechanical demands of the human heart may be sufficient to exceed this lower threshold for oxidant stress and promote the development of cardiac pathology.

Accumulating evidence suggested that Frataxin-deficient cells and tissues may experience impaired lipid metabolism. A recent study using Drosophila models of FRDA demonstrated a global accumulation of free fatty acid species as wells as specific accumulation of lipid droplets in glial cells (*31*). However, this study failed to distinguish between a potential impairment in lipid oxidation and an increase in lipid synthesis. Furthermore, a recent gene expression study of peripheral blood lymphocytes derived from human FRDA patients and heterozygous carriers for the GAA repeat expansion also provided evidence for rearrangements of lipid homeostasis (*32*). These studies emphasize that altered lipid biology may be a systemic factor of FRDA pathogenesis, a notion that has critical implications for both cardiac and mitochondrial function. An increasing

body of evidence generated predominantly through studies of type 2 diabetes indicates that there are systemic pathological consequences associated with impaired lipid homeostasis (*33*). These include widespread inflammation mediated by adipokines and toxic accumulation of oxidized lipid species and ceramides which are known to induce tissue damage and apoptosis, particularly in the human heart (*34*). The β-Oxidation of fatty acids occurs in the mitochondrial matrix, where it serves to generate acetyl-CoA for further oxidation through the TCA cycle. Fat is the predominant source of carbon fuel for the heart, deriving as much as much as 70% of the ATP necessary for contraction from fatty acid oxidation (*35, 36*). Importantly, a prolonged energetic shift away from fatty acid oxidation is a well-known characteristic of developing heart failure (*37*), suggesting that this may contribute to FRDA disease progression. Furthermore, impairments in fatty acid oxidation are known to occur in the skeletal muscle of patients with clinical evidence of respiratory chain dysfunction (*38*). Alternatively, a functional deficit of mitochondrial aconitase could promote increased mitochondrial efflux of citrate derived from the TCA cycle, which could serve as the carbon source for cytosolic lipid synthesis via ATP citrate lyase (*39*). These results potentially provide an indirect mechanism for impaired lipid metabolism in FRDA hearts. However, further work is necessary to determine what role, if any, impaired lipid homeostasis plays in FRDA.

There is emerging evidence that Frataxin deficiency may also impair the synthesis and maturation of cytosolic and nuclear-localized iron-sulfur cluster (ISC)-dependent proteins. Eukaryotic ISCs are generated in the mitochondrial

matrix via a complex 190 kDa protein complex composed of Frataxin, NFS1, ISD11, and ISCU as described earlier (*17*). The mitochondria-generated ISCs then translocate to the cytosol via the ABCB7 transporter and are incorporated into apoproteins via the cooperative functions of Cfd1, Nbp35, IOP1, CIAO1, and MMS19 (*40*). Several proteins involved in DNA replication and repair, telomere maintenance, and methionine biosynthesis require an intact ISC and are deficient when components of the cytosolic ISC protein assembly machinery are disrupted (*41*). Thus, since extra-mitochondrial ISC proteins also depend on Frataxin for their proper assembly, impairments in the aforementioned processes may also contribute to disease pathogenesis in Friedreich's ataxia.

A multitude of altered cellular processes have been implicated in the pathogenesis of FRDA over the seventeen years since the causal gene defect was discovered in 1996. The most convincing evidence indicates that lack of Frataxin causes respiratory chain defects and consequent ATP deficiency and mitochondrial iron-loading. As research progresses, the additional arguments for increased oxidative stress, altered lipid homeostasis, and defects in extra-mitochondrial ISC proteins may become persuasive and influence the rational develop of treatments for FRDA.

FRDA Disease Manifestations, Clinical Management, and Emerging Treatments

The cellular consequences of Frataxin deficiency which include ATP deficiency, iron accumulation, increased ROS, altered lipid metabolism, and

perhaps genomic instability, are all likely to contribute to the clinical

manifestations of FRDA. FRDA patients typically present with symptoms of ataxic

gait, fatigue and low energy during their second decade of life, but sometimes

before 10 years of age. The age of disease onset and length of disease duration

are inversely correlated with the size of the shorter GAA repeat expansion, with

longer expansions being associated with earlier age of onset and shorter lifespan

(*42*). Consistent with the progressive and degenerative nature of FRDA, the

majority of patients soon lose ambulatory capability and develop hypertrophic

cardiomyopathy within 10-15 years of onset. A subset of patients will also

develop scoliosis, dysarthria, and diabetes (*42*).

Consistent with loss of motor coordination, post-mortem analysis of FRDA

patient nervous tissue has demonstrated smaller dorsal root ganglia, a thinning

of the spinal cord, and fiber loss in the spinocerebellar and corticospinal tracts.

The dentate nucleus is also typically atrophied (*42*). Histochemical observations

of the dorsal root ganglia demonstrate reduced size of subscapular nerve cells,

cytoplasmic destruction, and an accumulation of immunoreactive ferritin and

ferroportin (*42, 43*). The latter observations are believed to be caused by iron-

loading. The dorsal spinal roots show thinning of myelin sheaths while the

dentate nucleus exhibits loss of large neurons.

Upon post-mortem analysis, the gross pathology of the heart generally

shows hypertrophy, thickening of the left ventricular wall, right ventricular wall

and interventricular septum (*42*). As the disease progresses, the left ventricle

may become dilated (*44*). FRDA hearts can weigh 2-2.5 times the average for

adult men and women (*42*). Histochemical and ultrastructural observations of the

FRDA heart show an increase in myocyte size, excessive endomysium,

scattered iron-reactive inclusions, and proliferation of abnormal mitochondria

(*42*). Additionally, the pancreas of FRDA patients who develop diabetes exhibits

islet loss and shrinkage (*42*).

Although FRDA is usually considered a neurodegenerative disorder, the

most common cause (>60%) of patient mortality is congestive heart failure or

other heart-related pathology such as arrhythmia (*42, 45*). Despite the serious

cardiac manifestations of FRDA, this aspect of the disease is often overlooked

both in the clinic and in basic research and requires special attention. The most

common finding among FRDA patients who have cardiac involvement is

concentric left ventricular hypertrophy. To better understand and diagnose FRDA

heart disease, a recent study grouped 205 FRDA patients based on the severity

of their cardiomyopathy which was determined by increasing left ventricular mass

measured by cardiac MRI. The investigators found that echocardiography

measurement of end-diastolic thickness of the posterior wall and interventricular

septum was strongly correlated (*P*<.049) with myocardial mass, making this

parameter a reliable and easily accessible measure of left ventricular hypertrophy

(*46*). Left ventricular ejection fraction measured by MRI was employed as a

measurement of cardiomyopathy. In the subset of patients having the greatest

myocardial mass, there was an observed decrease in the thickness of the

posterior wall and ventricular septum, which likely indicates a progression toward

myocardial thinning and dilation which is often accompanied by fibrosis (*46*). The

authors observed no correlation between cardiac parameters and neurological function. In conclusion, the authors recommended performing both cardiac MRI and echocardiography of each FRDA patient as early as possible in the disease course to establish a baseline from which disease progression and potential treatment options can be assessed.

There currently are no effective or targeted approved treatments for mitochondrial disorders with respiratory chain involvement with the exception of Coenzyme Q deficiency which can be treated well with dietary Coenzyme Q. The general approach to treating mitochondrial disease is the administration of antioxidants which are intended to mitigate cellular damage caused by aberrant production of reactive oxygen species. Other approaches include identifying enzyme deficiencies associated with the primary mitochondrial defect and supplementing the patient with the relevant intermediary metabolite. These approaches are untargeted and serve to mitigate the downstream consequences of the primary deficiency and, in most cases, have little therapeutic efficacy. Similarly, approved treatments for Friedreich's Ataxia are sorely lacking. Antioxidant treatment in FRDA has demonstrated moderate biochemical improvement but virtually no clinical improvement (*47*). The observed accumulation of mitochondrial iron in FRDA has led to the development of membrane permeable iron-chelating drugs such as deferiprone, which have demonstrated promise in cellular models of Frataxin deficiency and, more recently, in FRDA patients used in combination with the coenzyme Q mimetic idebenone (*26, 48*). Nonetheless, the greatest improvements in neurologic and

cardiac parameters in response to treatments are typically experienced by younger FRDA patients, while older individuals are less responsive to these treatments. This age-dependent response to treatment most likely reflects the progressive and worsening nature of FRDA over time and strongly argues for early intervention with current or potential future treatment options as well as for newborn screening to identify FRDA patients as early as possible (*49*). There are several untargeted interventions for FRDA at various stages in the drug development pipeline (Figure 1.2). In addition to deferiprone and idebenone, the peroxisome proliferator activated receptor gamma (PPARλ) agonist pioglitazone has been advanced as a therapeutic candidate for FRDA after a gene expression array study in animal models concluded this pathway was dysregulated (*50*). Despite having advanced through phase II clinical trials, the PPARλ agonist approach has elicited some concern among FRDA researchers due to findings demonstrating an increased risk of congestive heart failure and ischemic events among diabetics using pioglitazone (*51*). Since cardiomyopathy is the most common cause of death in FRDA, pioglitazone could potentially exacerbate this condition and promote cardiac pathology. An additional therapeutic option being explored for FRDA is the administration of the hormone erythropoietin, which was shown to increase Frataxin expression in lymphocytes and fibroblasts *in vitro* (*52*). However, the results of subsequent clinical trials testing the effect of erythropoietin on Frataxin expression have been mixed (*53-55*). Future studies are needed to validate the potential use of erythropoietin in FRDA treatment.
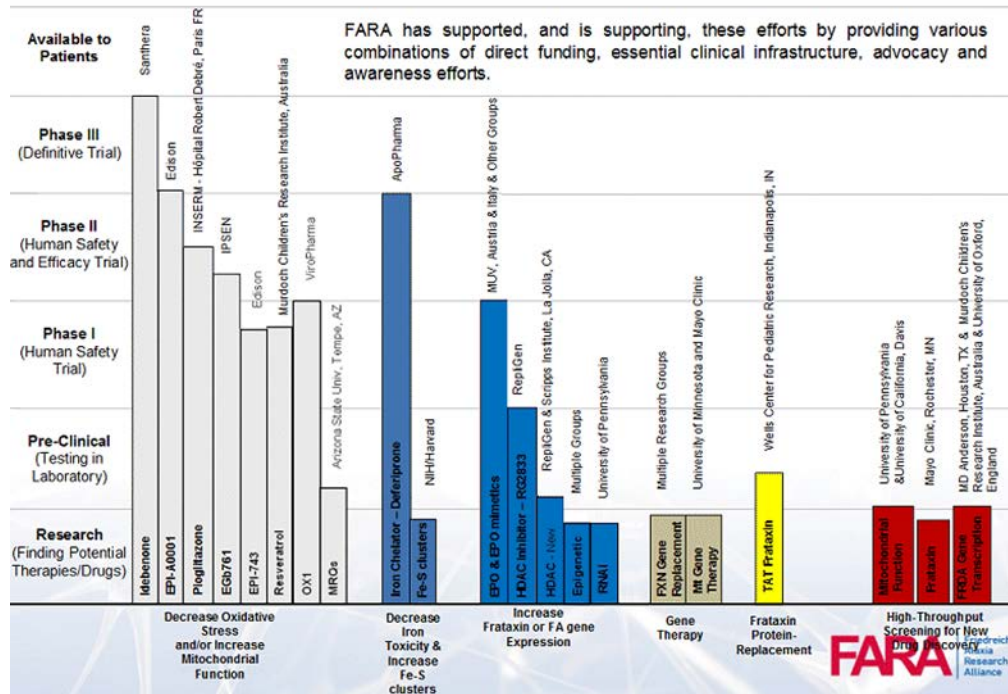
Figure 1.2 A summary of the different approaches to treating FRDA and their current stage of clinical development (Courtesy of the Friedreich's Ataxia Research Alliance, FARA, http://www.curefa.org/pipeline.html).

Despite the lack of an effective therapy for FRDA patients, recent progress has been made in the development of targeted therapies with the goal of either directly increasing Frataxin expression with small-molecule drugs or by replacing the deficient protein with exogenous, cell-penetrant Frataxin. Over the past several years, Dr. Joel Gottesfeld and colleagues from the Scripps Research Institute have approached the treatment of FRDA from an epigenetic standpoint. Initially, this group demonstrated that histone deacetylase (HDAC) inhibitors can alleviate transcriptional silencing of the *FXN* locus, which provided rationale for

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the development of more targeted and specific HDAC inhibitors in order to minimize potentially adverse global chromatin remodeling (*7*). This approach was later extended to show HDAC inhibitor-induced upregulation of Frataxin in humanized mouse models of FRDA as well partial reversal of an altered gene expression phenotype in cultured FRDA patient lymphocytes (*32, 56, 57*). More targeted HDAC inhibitors for increasing Frataxin expression have been developed, licensed, and are currently in Phase 1 clinical trials with Repligen Corporation (e.g. RG2833). Alternatively, Dr. R. Mark Payne of Indiana University has taken the protein replacement therapy approach to treating FRDA. Dr. Payne and colleagues have demonstrated that Frataxin can be attached to the cell-penetrant peptide transactivator of transcription (TAT-Frataxin) which can then enter cells, be correctly processed by mitochondrial processing peptidase, and can improve biochemical readouts of Frataxin deficiency (*58*). More importantly, TAT-Frataxin treatment can extend the lifespan and improve cardiac parameters in the NSE-Cre conditional mouse model of FRDA (*59*). Unlike the small molecule epigenetic approach, exogenous replacement of a mitochondrial protein using TAT also has the potential to treat many other mitochondrial diseases. The TAT-Frataxin concept is now in the early stages of licensing with venture capital and biotech companies for future development as a therapy for FRDA.

The ability to monitor treatment response with biomarkers is extremely important in the testing of new drug candidates. Currently, clinical trials for FRDA are hampered by the lack of reliable disease biomarkers indicative of an

improving or worsening patient condition. This problem also deters drug

companies from developing treatments for FRDA because they cannot determine

if their drug is effective. Some studies have suggested measuring serum levels of

malondialdehyde or oxidized DNA bases as biomarkers (*60, 61*). More recently,

a study showed that a serum biomarker traditionally used to measure cardiac

ischemia, Troponin I, is highly elevated in nearly 50% of FRDA patients, many of

whom were asymptomatic (*62*). These findings suggest that measurement of

serum cardiac troponin may have clinical utility as a biomarker in FRDA. Further

work on developing disease biomarkers in FRDA is needed in order to more

accurately monitor disease course and response to potential treatment options.

Mitochondrial Protein Acetylation[*]

Over millions of years, eukaryotic organisms evolved fine-tuned metabolic

mechanisms for orchestrating the conversion of diverse carbon substrates into

cellular energy. These mechanisms include changes in gene transcription,

allosteric regulation of metabolic enzymes by fluxing pools of intermediary

metabolites, and direct regulation by post-translational modifications (PTMs),

such as phosphorylation. One PTM, lysine acetylation, has been traditionally

studied in the context of nuclear histone modifications and is well-known to

influence changes in gene expression (*63*). However, recent proteomics surveys

have revealed that lysine acetylation is widespread cellular modification that is

particularly abundant on mitochondrial proteins, suggesting a new post-

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[*] **G. R. Wagner**, R. M. Payne, Mitochondrial acetylation and diseases of aging. *J Aging Res* **2011**, 234875 (2011).

translational mechanism for coordinating the metabolism of carbon sources (*64, 65*). Accordingly, several studies have demonstrated that reversible lysine acetylation can, by targeting key enzymes, modulate the activity of mitochondria-localized fatty acid β-oxidation, the tricarboxylic acid cycle (TCA), urea cycle, and oxidative phosphorylation in a nutrient-responsive manner (*66-69*). The reversible and nutrient-sensitive manner of many of these acetyl-modifications has strongly implicated the mitochondrial-localized members of the NAD^+-dependent deacetylases (sirtuins) as regulatory mediators of these fundamental metabolic processes (*70*). Sirtuins are also believed to be part of a genetic program influencing the development of age-related conditions such as heart disease, neurodegenerative disease, and cancer (*71*). Although seminal discoveries have been made in the basic biology of mitochondrial acetylation, an understanding of how acetylation states influence enzyme function and metabolic reprogramming during age-associated pathological states remains largely unknown.

This section will examine mammalian acetate metabolism and provide a brief history of lysine acetylation as a means to discussing more recent advances in mitochondrial acetylation biology and its potential role in the age-related conditions of heart failure and cancer, as well as its potential role in human longevity.

Acetyl-CoA and Metabolism

Acetate is an ancient energy precursor molecule of metazoan metabolism (Figure 1.3). In eukaryotic systems, the principal source of cellular acetate is generated by the mitochondrial processes of glucose-derived pyruvate oxidation, amino acid catabolism, and the oxidation of even-numbered fatty acyl chains. These distinct metabolic pathways are all capable of yielding the activated form of cellular acetate, or acetyl-CoA. Thus, acetyl-CoA represents a common convergence point for carbohydrate, amino acid, and lipid catabolism. How mitochondrial acetyl-CoA is utilized throughout the cell is heavily dependent on the cell type and the metabolic state of the organism. However, the primary function of acetyl-CoA throughout all cell types is as a carbon donor in the TCA cycle. Originally characterized by Hans Krebs and Albert Szent-Györgyi, the TCA cycle is a series of substrate oxidation and decarboxylation reactions that serve to liberate carbon dioxide and generate electron donors in the form of the reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (*72*). The coenzymes NADH and $FADH_2$, in turn, serve as high-energy electron donors which are oxidized by the respiratory chain complexes to generate the proton-motive electrochemical gradient that is ultimately responsible the formation of energy-rich ATP (*73*). Acetyl-CoA also serves as a vital component of anabolic cellular processes. In general, during a state of nutrient excess (fed state), a proportion of the excess acetyl-CoA generated within the mitochondria is exported into the cytosol in the form of citrate, where it is reconverted into acetyl-CoA by ATP-citrate lyase (*74*). This

cytosolic acetyl-CoA can then participate in *de novo* synthesis of fatty acids and

sterols that are vitally important for the formation and maintenance of lipid
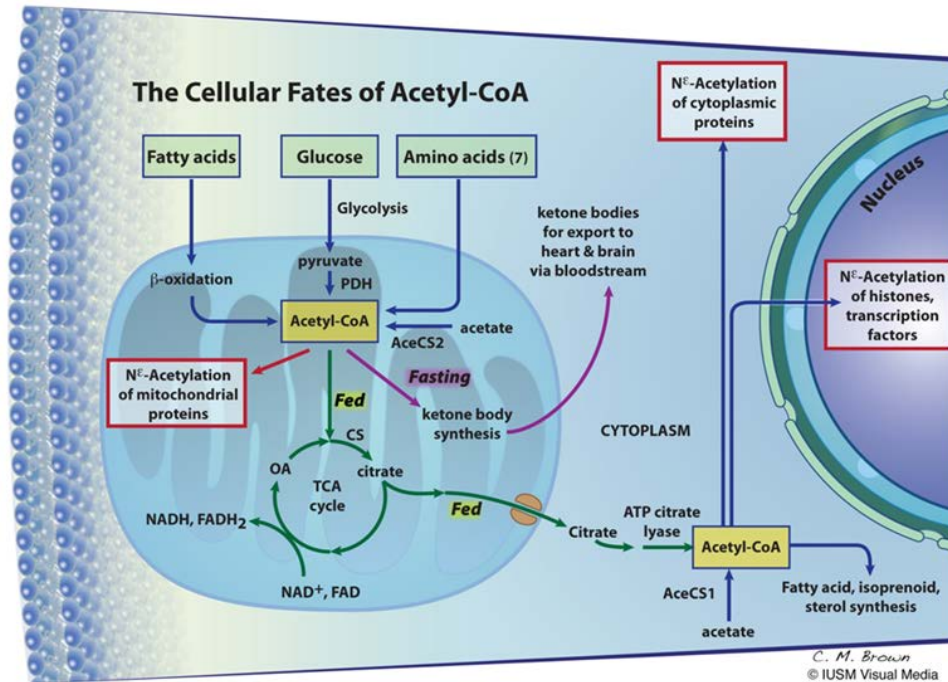
Figure 1.3 Cartoon schematic summarizing acetyl-CoA metabolism in a mammalian cell (*39*). Please see section entitled "Acetyl-CoA and Metabolism," for a detailed description of figure. Abbreviations: PDH, pyruvate dehydrogenase complex; AceCS2, acetyl-CoA synthetase 2; CS, citrate synthase; OA, oxaloacetate; AceCS1, acetyl-CoA synthetase 1.

membranes, as well as the synthesis of steroid hormones, triacylglycerols,

cholesterol, and fat soluble vitamins (*75, 76*). Lipogenesis primarily occurs in

hepatocytes and adipocytes whereas cholesterol synthesis primarily occurs in

hepatocytes. In contrast to the fed state, during a nutrient-depleted state, the liver

uses TCA cycle intermediates for gluconeogenesis which depletes oxaloacetate

and limits the entry of acetyl-CoA into the TCA cycle. As the liver continues to

oxidize fatty acids for fuel, acetyl-CoA begins to accumulate and is converted into the transport forms of acetate known as ketone bodies. Ketone bodies are then used as an alternative fuel source in the brain, heart, and skeletal muscle during fasting or glucose-scarce conditions (*77*). Free cytoplasmic and mitochondrial acetate can also be converted into acetyl-CoA via the ATP-dependent mechanisms of acetyl-CoA synthetase 1 and 2, respectively (*78, 79*). Interestingly, acetyl-CoA synthetase 2 (AceCS2) was recently found to be an enzyme critically involved in thermogenesis during fasting conditions (*80*). Through its well-established roles in the generation of cellular energy and as a macromolecular building block, acetyl-CoA stands as a critical chemical intermediate that is inextricably linked to cellular energy homeostasis. However, the fundamental and ancient link between acetyl-CoA, mitochondria, and metabolism is underappreciated in the context of acetyl-CoA's exciting and dynamic use as the acetyl group donor for acetyl-lysine post-translational modifications. Interestingly, a recent study highlighted this often overlooked aspect of acetyl-CoA metabolism by demonstrating that the metabolic enzyme ATP-citrate lyase, in addition to its previously mentioned roles in lipid and steroid synthesis, also regulates global patterns of histone acetylation and corresponding changes in gene expression (*81*). Therefore, it is nutrient availability and metabolism that largely determine the cellular pool of acetyl-CoA available for acetyl-lysine protein modifications.

N$^\varepsilon$- acetylation

Acetylation occurring on the epsilon-amino group of lysine residues (N$^\varepsilon$-acetylation) was discovered on histone proteins purified from calf thymus over forty years ago (*82*). The ensuing studies of N$^\varepsilon$-acetylation focused almost exclusively on histone substrates (*83-85*). It was not until 1996 that Taunton *et al.* purified the first histone deacetylase enzyme (*86*). This important discovery quickly expanded research interest in the field of histone acetylation, and in the past 17 years has led to the discovery of numerous enzymes that catalyze the addition and removal of acetyl groups, termed histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. The dynamic and opposing functions of HATs and HDACs form the basis for the transcriptional modulation of chromatin and the histone code hypothesis (*63, 87*). Due their role in global cellular processes of transcription, HDACs have become attractive chemotherapeutic targets. Vorinostat (suberoylanilide hydroxamic acid) is an FDA approved HDAC inhibitor that is currently being used for the treatment of cutaneous T-cell lymphomas and there are a number of related compounds in clinical trials for other neoplastic indications (*88*).

Owing to the highly conserved nature of HATs and HDACs across multiple eukaryotic species, they are commonly classify according to their homologs in yeast (*89*). There are three primary families of HATs. These include the MYST family, the Gcn-5-related N-acetyltransferases (GNATs) and the E1A-associated protein of 300 kDa/CREB-binding protein (p300/CBP) family (*90*). HDACs are broadly classified into the Rpd3/Hda1 family of deacetylases, and the silent

information regulator-2 (Sir2)-like NAD$^+$-dependent deacetylases/ mono-ADP-ribosyltransferases, or sirtuins. The Rpd3/Hda1 family are further grouped into classes I, II, and IV, while the sirtuins represent class III deacetylases (*71, 91*). Due to the histone-centric nature of the founding studies on acetylation, these acetylase and deacetylase enzymes are traditionally referred to as HATs and HDACs. However, studies in the past ten years have established that the so-called HATs and HDACs have multiple non-histone and extra-nuclear targets including, among many others, the tumor suppressor p53, the oncogene β-catenin, and the molecular chaperone HSP90 (*92-95*). Accordingly, these enzymes are now more generally referred to as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs).

Biochemically, the positively charged ε-amino group of lysine residues is important for protein stability via hydrogen bonding with nearby amino acid residues and can also serve as a base during enzyme catalysis (*96*) (Figure 1.4). The covalent enzymatic addition of an acetyl moiety to the ε-amino group on a lysine residue effectively neutralizes its native positive charge, which changes its propensity to interact with nearby residues, other proteins, or can alter the activity of an enzyme. In its well-established chromatin context, acetylation neutralizes positively charged lysine residues on histone tails, thereby reducing its charged interactions with other chromatin elements and inducing a "loose" conformation that is amenable to transcription factor entry and binding (*63*). In another context, acetylation of lysine 88 on the enzyme ornithine transcarbamylase (OTC) changes its affinity for its substrate, carbamoyl phosphate, and negatively
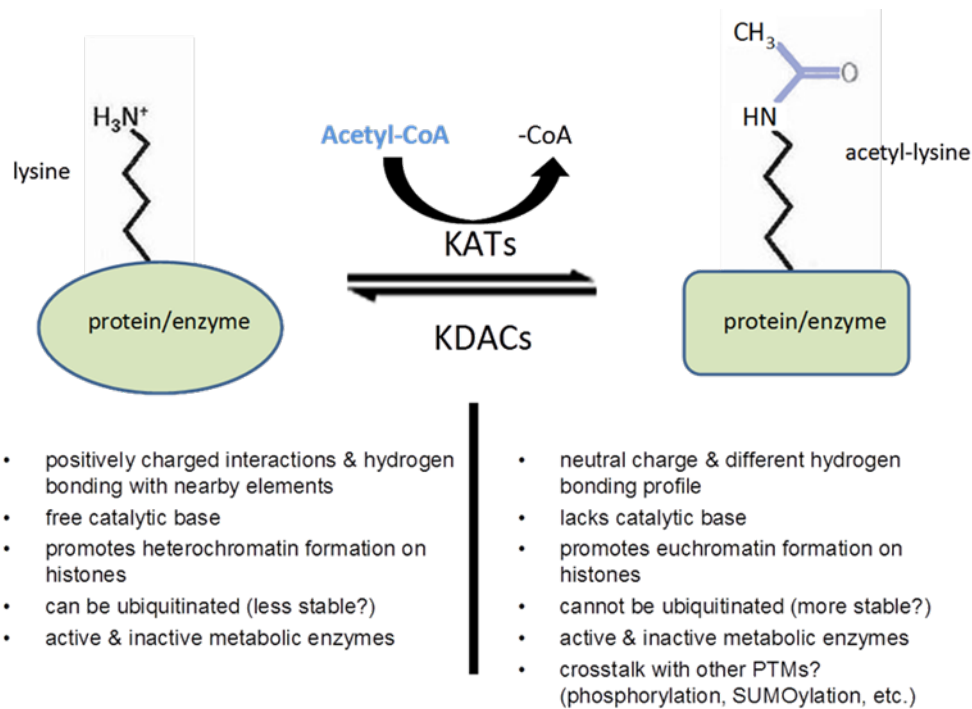
Figure 1.4 The biochemistry of lysine acetylation. Changes in protein function and stability known to be associated with lysine acetylation (*39*). Abbreviations: KATs, lysine acetyltransferases; KDACs, lysine deacetylases.

regulates the detoxification of ammonia in the urea cycle (*97*). Additionally,

acetylation of lysine 685 on STAT3 promotes its stable dimerization and

subsequent function as a transcriptional activator (*98*). The ε-amino groups of

lysine residues are also the targets of a number of other post-translational

modifications including methylation, ubiquitination, SUMOylation, and

NEDDylation. These other modifications may confer their own unique properties

to proteins and protein functions that could be coordinated with

acetylation/deacetylation. Ubiquitination of lysine residues, in particular, is known

cooperate with acetylation to regulate the proteolytic degradation of cellular

proteins (*99*). It has also been proposed that acetylation may also act in concert

with other distinct post-translational modifications to achieve a broad spectrum of functional protein outcomes that has been termed the "protein modification code," analogous to the "histone code" for transcriptional regulation (*100*). In general, it has become clear in recent years that N^ϵ- acetylation is a widespread and functionally diverse post-translational modification.

NAD$^+$-dependent deacetylases (Sirtuins)

In their traditional nuclear context, KATs catalyze the acetyl-CoA dependent addition of acetyl groups to disordered histone tails – a mark that generally induces a "loose" and transcriptionally active conformation of chromatin. In contrast, the traditional functional understanding of KDACs holds that they catalyze the H_2O-dependent removal of acetyl groups from histone tails to induce "tight" and transcriptionally repressed chromatin (*87*). This well-established paradigm for transcriptional modulation was broadened with the discovery of the mechanistically distinct sirtuin class of deacetylases/mono-ADP ribosyltransferases.

There are seven mammalian sirtuin enzymes. The nuclear-localized family members consist of SIRT1, SIRT6, and SIRT7, while SIRT2 is concentrated in the cytosol (*71*). SIRT3, SIRT4, and SIRT5 exhibit mitochondrial localization (*71, 101, 102*). Together, the sirtuin family of KDACs are implicated in the regulation of a broad scope of biological processes including, but not limited to, transcriptional repression, development, apoptosis, DNA repair, cellular stress responses, and metabolism (*103-107*). Sirtuins are unique among the four

families of mammalian KDACs in that they catalyze the removal of acetyl groups in a fashion that is dependent on the cellular co-enzyme NAD^+. The reduced form of NAD^+, NADH, is generally produced during cellular catabolic processes that serve to derive energy from nutrients, whereas the oxidized form, NAD^+, accumulates within cells during nutrient scarce conditions. Thus, the ratio of the reduced form to the oxidized form, or NAD^+/NADH, is indicative of the overall cellular energy state. Sirtuin deacetylase activity is responsive to this measure of cellular energetic balance and require NAD^+ as the acceptor of an acetyl group from a target acetyl-lysine residue or for protein ADP-ribosylation (*108*). The NAD^+-dependent deacetylase reaction yields a deacetylated target protein, 2'-O-acetyl-ADP-ribose, and nicotinamide, which can then act to inhibit the sirtuin reaction mechanism (*109*). In general, when the cellular NAD^+/ NADH ratio is elevated during times of exercise, nutrient-restriction or outright fasting, sirtuins are presumed to be more actively deacetylating their target proteins, whereas during a fed or basal state, NADH predominates and sirtuins are less active. It is via their nutrient and stress-sensing capacity to modify transcriptional landscapes and modulate enzyme function that sirtuins are believed to mediate the healthful benefits of caloric restriction – the only natural means of consistently extending lifespan and delaying the onset of age-related pathologies such as cancer in mammalian systems (*70, 110-113*). It is the possibility that the aging process is not simply a consequence of time, but rather a program encoded by a set of genes, that has excited the research community and has made the sirtuins highly attractive therapeutic targets for diseases of aging (*114*).

Mitochondrial sirtuins and N$^\varepsilon$-acetylation

Mitochondria are key organelles for intermediary metabolism as they coordinate the conversion of cellular carbon sources into useable energy in the form of ATP. The metabolic processes culminating in ATP synthesis are well-characterized and are regulated by various mechanisms including nutrient availability, phosphorylation, allosteric mechanisms, reactive oxygen species, and divalent cations such as calcium and magnesium (*115-118*). In light of the established understanding that mitochondria are the primary cellular generators of the acetyl-CoA necessary for enzymatic acetylation, it is surprising that N$^\varepsilon$-acetylation within the mitochondria remained virtually unstudied until the last decade. The discovery that the sirtuin family members SIRT3, SIRT4, and SIRT5 localize to the mitochondrial matrix has strongly suggested that acetylation could also play an important regulatory role within this organelle (*101, 102*). An early study by Shi, *et al.* supported this suggestion by demonstrating that SIRT3 regulates adaptive responses to cold exposure in brown adipose tissue by increasing the expression of mitochondrial uncoupling protein-1 (UCP1) and thereby increasing mitochondrial respiration (*119*). Soon after this study was published, Schwer, *et al.* and Hallows, *et al.*, independently confirmed that mammalian SIRT3 directly regulates the activity of the mitochondrial protein acetyl-CoA synthetase 2 (AceCS2) via NAD$^+$-dependent deacetylation (*120, 121*). These findings demonstrated an ancient metabolic regulatory mechanism conserved from the prokaryotic bacterium *Salmonella enterica* to mammals (*122*). Taken together, these early studies not only confirmed reversible N$^\varepsilon$-

acetylation as an important regulatory mark within mitochondria, but also implicated the sirtuins as key nutrient-responsive modulators of the functional changes acetylation confers upon its target proteins.

The initial studies of mitochondrial acetylation gave way to global proteomics surveys. A 2006 proteomics survey conducted by Kim, *et al*. revealed that over 20% of liver mitochondrial proteins and enzymes are acetylated, and that many change acetylation states in response to acute fasting (*64*). Furthermore, enzymes of the major mitochondrial carbon conversion pathways such as the TCA cycle and β-oxidation were found to be particularly enriched in acetyl-modifications. These findings suggested that reversible acetylation could serve as a finely-tuned mechanism for globally regulating the use and conversion of carbon energy sources. The authors also demonstrated that a number of the mitochondrial enzymes they found to be acetylated have known implications in processes of mammalian aging and longevity, such as manganese superoxide dismutase (MnSOD) and NADH: ubiquinone oxidoreductase (respiratory complex I) (*64*). Complementing the discovery that global acetylation profiles can change in response to nutrient availability, Schwer, *et al.* showed that global mitochondrial acetylation profiles change in multiple organ systems in response to long-term caloric restriction (CR) (*123*). Surprisingly, this study found that the vast majority of mitochondrial enzymes exhibited increases in acetylation during caloric restriction, which counters the belief that sirtuin-mediated deacetylation mediates the beneficial effects of caloric restriction. This finding could suggest that mitochondrial sirtuin-mediated deacetylation plays a relatively minor role in

the metabolic changes that accompany caloric restriction and that there are more complex and undiscovered adaptive mechanisms at play.

In addition to published acetylation changes during caloric restriction, ongoing data from our investigations demonstrate that mitochondrial acetylation profiles in the liver, kidney, and brain are different between young and aged mice, suggesting that acetylation could mediate age-associated changes in metabolism. Similarly, Wang, *et al.* and Zhao, *et al.* confirmed the regulatory importance of acetyl-modifications in metabolism and in the coordination of cellular carbon utilization (*65, 124*). These proteomics surveys have exposed the prevalence of acetylation within mitochondria and reinforced its importance as a regulatory modification. Ongoing studies of acetylation changes with age are likely to reveal this as an important regulatory mechanism for mitochondrial biology.

Further focused investigations of acetylation within mitochondria have concentrated on the role of mitochondrial sirtuins. By generating SIRT3, SIRT4, and SIRT5 systemic knockout mouse models, Lombard, *et al.* demonstrated that mice lacking SIRT3 exhibit global hyperacetylation of mitochondrial proteins in liver and brown adipose tissue, whereas the acetylation states in mice lacking SIRT4 and SIRT5 were relatively unchanged (*125*). This evidence suggested that SIRT3 primarily functions as a deacetylase and potentially targets a wide range of mitochondrial proteins, whereas the deacetylase activities of SIRT4 and SIRT5 are much more restricted. Early characterization of SIRT3 null mice indicated that they were phenotypically unremarkable, despite a significant reduction in their

liver, heart, and kidney ATP content associated with hyperacetylation and

reduced activity of mitochondrial respiratory complex I (*69*). A more thorough

investigation, however, revealed that SIRT3$^{-/-}$ mice display hepatic lipid

accumulation during fasting resembling human disorders of fatty acid oxidation

(*66, 126*). This study found that SIRT3 directly deacetylates and activates long-

chain acyl-CoA dehydrogenase (LCAD), illustrating a critical mechanism for this

sirtuin in the metabolic adaptations to fasting and nutrient restriction (*66*).

Consistent with SIRT3's role in oxidative metabolism, Shulga, *et al*.

demonstrated that SIRT3 can stimulate oxidative phosphorylation and a shift

away from glycolysis via inactivation of cyclophilin D, which consequently

destabilizes the association of hexokinase II with the mitochondria (*127*).

Furthermore, mitochondrial isocitrate dehydrogenase (IDH2) is deacetylated and

activated by SIRT3, thereby regulating a key step in the TCA cycle (*67*).

Additional studies have exposed SIRT3 as a regulator of protein translation via

deacetylation of the mitochondrial ribosome and in the activity of respiratory

complex II (succinate dehydrogenase) (*128, 129*). It is now clear that SIRT3 can

influence the activity of oxidative phosphorylation, fatty acid oxidation, and the

TCA cycle, positioning this particular sirtuin as a major player in the regulation of

intermediary metabolism.

Studies of mitochondrial SIRT4 and SIRT5 are less extensive than those

for SIRT3. Earlier characterization of mammalian SIRT4 revealed that it chiefly

functions as an NAD$^+$-dependent mono-ADP-ribosyltransferase that specifically

ADP-ribosylates and inactivates mitochondrial glutamate dehydrogenase (GDH),

thereby regulating amino acid-induced insulin secretion in pancreatic β cells
(*130, 131*). Later studies also identified GDH as a target of SIRT3-mediated
activation via deacetylation, but potential cross-talk with SIRT4-mediated ADP-
ribosylation has not been explored (*67, 125*). Recently, it was demonstrated *in
vivo* that adenoviral-mediated shRNA knockdown of SIRT4 in mouse liver
increases the expression of genes involved in fatty acid metabolism (*132*).
Together, these lines of evidence suggest SIRT4 could play an important role in
the disruption of lipid homeostasis associated with insulin resistance in type 2
diabetes. Further characterization of mitochondrial SIRT5 has shown that it
regulates the first step in the detoxification of ammonia during the urea cycle via
deacetylation-mediated activation of carbamoyl-phosphate synthetase 1(*68*).
Accordingly, SIRT5$^{-/-}$ mice develop hyperammonemia during metabolic states
demanding greater protein catabolism such as fasting and caloric restriction (*68,
133*). Another investigation has suggested SIRT5 can deacetylate cytochrome *c*,
a protein involved in mitochondrial respiration and apoptosis (*67*). The functional
importance of this relationship, however, has not been determined. SIRT5, on the
other hand, exhibits weak deacetylase activity and recent *in vitro* studies suggest
that SIRT5 functions primarily as a protein de-succinylase and de-malonylase
(*134*). Future studies of mitochondrial SIRT4 and 5 are likely to elucidate their full
regulatory capacity in response to various cellular stresses and stimuli.

Interestingly, other studies have implicated reversible mitochondrial
acetylation in mechanisms of cell growth and survival. An early investigation of
SIRT3's function in an epithelial cancer cell line indicated that it is required for

JNK-regulated cell death via silencing the apoptotic regulator B-cell lymphoma 2 (bcl-2) (*135*). In contrast to its role in epithelial cancer, SIRT3 was shown to serve a pro-survival function in cardiomyocytes via deacetylating Ku70 and promoting the sequestration of the pro-apoptotic protein Bax (*104*). In addition to its apoptotic functions, SIRT3 was more recently demonstrated to be a bona fide tumor suppressor by Kim, *et al.* (*136*). This study showed SIRT3^{-/-} mouse embryonic fibroblasts display reduced antioxidant defenses, which result in a lower threshold for tumorigenesis – a finding that confirmed earlier work that established SIRT3's involvement in the transcriptional regulation of antioxidant genes (*137, 138*). Furthermore, the transformation-permissive effect of SIRT3 ablation can be reversed upon the addition of exogenous MnSOD (*136*). A different study conducted by Yang, *et al.*, established that the levels of mitochondrial NAD$^+$ were a critical determinant to cell survival (*139*). Mitochondrial NAD$^+$ levels were shown to be regulated by the rate-limiting enzyme in the mammalian NAD$^+$- salvage pathway, nicotinamide phosphoribosyltransferase (Nampt). Importantly, the authors demonstrated that NAD$^+$-responsive mitochondrial SIRT3 and SIRT4 were required for improved cell survival when mitochondrial NAD$^+$-levels rise under conditions of fasting and induced genotoxic stress (*139*). Recently, two independent approaches also implicated SIRT3 in the regulation of p53-mediated growth arrest (*140, 141*). Taken together, these studies indicate mitochondrial SIRT3 is a critical mediator of cell growth and survival which may hold therapeutic implications in the treatment of various cancers.

There are also several interesting acetylated mitochondrial proteins involved in cellular stress responses and apoptosis on which the effect of reversible acetylation has not been studied. These proteins include apoptosis-inducing factor (AIF) responsible for DNA fragmentation during apoptosis, cytochrome *c* which forms an important component of the apoptosome, and voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT), both of which are believed to form the mitochondrial permeability transition pore during apoptosis (*64, 142*). MnSOD is also known to be reversibly acetylated which may influence the detoxification of mitochondrial reactive oxygen species and the overall tendency of a cell to undergo apoptosis (*64, 123*). Furthermore, all of the previously mentioned mitochondrial proteins are reversibly acetylated in a nutrient-sensitive manner, suggesting that the apoptotic functions of these proteins may be regulated by cellular energy status (*64*). The study of mitochondrial acetylation states in processes of cell death and survival represents an entirely open field of study that is rife with intriguing biological questions that could provide a new layer of understanding to the mechanisms and regulation of apoptosis and cell growth.

The majority of the studies in the field of mitochondrial acetylation thus far have focused on the mitochondrial-localized members of the sirtuin family of deacetylases, while the search for the counterbalancing mitochondrial acetyltransferase(s) so far has been unsuccessful. Although it is possible that certain nuclear-encoded, mitochondrial-targeted proteins acquire acetylation marks prior to mitochondrial import, the confirmed acetylation of the

mitochondrial-confined ATP synthase subunit 8 protein makes the existence of a

mitochondrial acetyltransferase(s) possible (*64, 142*). The marked enrichment of

acetyl-modifications occurring on enzymes involved in major processes such as

β-oxidation and redox chemistry (64% and 44%, respectively) over the total pool

of acetylated mitochondrial proteins (22%), suggests finely-tuned enzymatic

mechanisms for regulating carbon flux within mitochondria (*64*). Furthermore, the

amino acid sequence motifs favoring acetylation within mitochondria differ from

those of the nucleus and cytosol, suggesting a mitochondrial-specific

acetyltransferase(s) possessing its own distinct set of substrates (*64*). Despite

the evidence for a mitochondrial acetyltransferases(s), it is also important to

consider the possibility of non-enzymatic acetylation occurring in response to

changes in the overall mitochondrial acetyl-CoA pool. Indeed, non-enzymatic

acetylation is known to occur on histone substrates and may account for the

unexpected increases in mitochondrial acetylation in response to caloric

restriction (*123, 143*). The discovery of the mitochondrial acetyltransferases(s) or

alternative acetylation mechanisms will provide a critical step toward a more

complete understanding of mitochondrial acetylation biology and its implications

for disease states.

Mitochondrial acetylation and heart failure

        The recently understood prevalence of acetyl-modifications within the

mitochondria that can potentially modulate processes of intermediary metabolism

suggests that altered acetylation could potentially contribute to age-associated

disease states in which there are recognized alterations in mitochondrial carbon utilization, such as heart failure and cancer.

The unfortunate progression from increased cardiac workload to pathological cardiac hypertrophy and, ultimately, heart failure, occurs when the long-term metabolic demands of the body exceed the ability of the pumping heart to meet them. Although there are a number of human conditions capable of leading to heart failure including renal insufficiency, high blood pressure, and cardiac arrhythmias, the leading risk factor is increasing age (*144*). The role of mitochondrial dysfunction in heart failure and the aging process in general is well-established. Mitochondrial disorders are often characterized by myopathies of the heart and skeletal muscle, as these highly energetic organs are heavily dependent on mitochondria-derived energy generation (*145*). Furthermore, cardiomyopathy and heart failure in the general population are often regarded as diseases of impaired energy homeostasis associated with a depletion of the cardiomyocyte ATP and creatine pools (*37, 146*). Interestingly, mice lacking the predominant mitochondrial NAD^+-dependent deacetylase SIRT3 also exhibit depleted cardiac ATP levels and further display global hyperacetylation of mitochondrial proteins in liver and brown adipose tissue (*69, 125*). These data suggest a role for mitochondrial SIRT3 dysfunction in the pathophysiology of heart failure. Several additional studies also stand in support of this notion. As discussed earlier, Sundaresan, *et al.* have shown that SIRT3 promotes cardiomyocyte cell survival during genotoxic and oxidative stress (*104*). This group has also revealed that SIRT3 transgenic mice are resistant to agonist-

induced cardiac hypertrophy via SIRT3-mediated upregulation of antioxidant genes (*138*). Moreover, exogenous administration of NAD^+ into the hearts of mice was found to abrogate agonist-induced cardiac hypertrophy in a SIRT3-dependent manner (*147*). Similarly, transgenic mice harboring cardiac-specific overexpression of the NAD^+-salvaging enzyme Nampt display reduced infarct size following ischemia-reperfusion injury, raising the possibility that the cardioprotective effect of increased NAD^+ is mediated by the mitochondrial sirtuins (*148*). Pathophysiological cardiac remodeling and heart failure in humans are also known to develop in response to alterations in the renin-angiotensin system (RAS) (*149*). One of the RAS signaling proteins, angiotensin II, is often used to induce hypertension and cardiac hypertrophy in experimental animals. Interestingly, ablation of the angiotensin II type I receptor in mice reduces cardiac injury, increases lifespan, and upregulates the expression of SIRT3 and its upstream regulator nampt in the kidney (*150*). Collectively, these studies implicate the mitochondrial deacetylase SIRT3 as a major cardioprotective enzyme, the modulation of which may hold therapeutic promise in the treatment of cardiac disease.

Mitochondrial oxidative phosphorylation is responsible for generating over 90% of the energy-rich ATP in the adult human heart (*35, 151*). Under basal conditions, as much as 70% of this ATP is derived from fatty acid β-oxidation and the remainder is generated from the oxidation of glucose, lactate, and ketone bodies (*36*). The energetic substrate preferences of the heart can change in response to acute stimuli or stressors such as exercise, energy demand,

hormonal regulation, ischemia, and substrate availability (*36, 152*). Thus, the

healthy heart is a versatile and adaptive consumer of energy substrates. The

failing heart, however, experiences complex alterations in energy metabolism

and substrate utilization that are incompletely understood (*153*). Studies on

rodent and canine models generally indicate that the failing myocardium suffers

downregulation of mitochondrial respiratory chain complexes, decreased oxygen

consumption and ATP generation, decreased flux through the creatine kinase

system, and impaired fatty acid oxidation (*36, 153-156*). Many have proposed

targeting these metabolic rearrangements and altering substrate utilization as a

therapeutic strategy to treat heart failure (*157*). Interestingly, numerous

mitochondrial enzymes of the respiratory chain and involved in the oxidation of

glucose and fat carry one or more acetylation marks that are reversible in a

nutrient-sensitive manner. These enzymes include respiratory complexes I, II,

and V, pyruvate dehydrogenase, several acyl-CoA dehydrogenases, and

carnitine palmitoyltransferase 1 and 2, among many others (*64, 123, 142*). It has

also been demonstrated that the mitochondrial deacetylase SIRT3 can directly

promote oxidative metabolism via deacetylation-mediated activation of

respiratory complex 1, LCAD, and cyclophilin D as discussed earlier (*66, 69,

127*). The notion that acetylation within the mitochondria can modulate the

activity of metabolic enzymes implies that there may be altered acetylation states

within the mitochondria and that they could contribute to the pathophysiology of

heart failure. More importantly, if this is true, devising strategies that directly

target mitochondrial acetylation may provide therapeutic benefit in pathological rearrangements of cardiac energy metabolism.

Mitochondrial acetylation and cancer

In 1924, Otto Warburg made the seminal observation that many cancer cells prefer to metabolize glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation (*158*). This phenomenon, known as the "Warburg Effect" or aerobic glycolysis, was hypothesized by Warburg to be a defect in the mitochondrial metabolism of tumor cells (*159*). In the roughly 70 years following its discovery, little progress was made in uncovering the biochemical mechanisms underlying the propensity of tumor cells to prefer aerobic glycolysis. The last 15 years of oncology research, however, has experienced a renewed interest in understanding the Warburg Effect and a number of important discoveries have been made. Notably, it has been observed that tumor cell lines almost exclusively express the embryonic isoform of the glycolytic enzyme pyruvate kinase (PKM2), which contributes to the glycolytic preference and lactate production observed in cancer cells (*160, 161*). Recent work conducted by Vander Heiden, *et al*. has even provided early evidence of an alternative carbon utilization pathway in tumors expressing PKM2 (*162*). Further work on tumor metabolism has revealed that malignant cells also consume large amounts of glutamine in an effort to replenish the TCA cycle intermediates necessary for a high rate of macromolecular synthesis that is characteristic of rapidly proliferating cells (*163*). This "glutamine addition" can

partially be explained by induction of a transcriptional program that favors

glutaminolysis via the activation of the proto-oncogene Myc, which can occur in

many forms of human cancers including neuroblastoma, lymphoma, and small

cell lung cancer (*164-168*). Together, these studies and the recent revitalized

interest in cancer bioenergetics have unveiled some key mechanisms underlying

the remarkable ability of transformed cells to proliferate. Despite these recent

advances, there is still much we do not understand about a tumor cell's aptitude

for reprogramming metabolism to meet its rapid growth demands and this

represents an active area of investigation.

Orchestrating malignant changes in cellular behavior often occurs at the

level of post-translation regulation. Indeed, many cancers are characterized by

mutations in, or altered activity of, certain kinases including tumor suppressor

LKB1, *Src*, phosphosphoinosital-3-kinase (*PI3K*), and the mutant *BCR-ABL*

fusion kinase, which often occurs in chronic myelogenous leukemia (CML) (*169-*

172). Additionally, there is an appreciated role for cancer-associated

transcriptional changes mediated by nuclear KATs and KDACs (*88, 173*).

Interestingly, the predominant mitochondrial deacetylase SIRT3 has recently

been reported to function as a tumor suppressor (*136*). This work demonstrated

that $SIRT3^{-/-}$ fibroblasts exogenously transformed with the oncogenes Myc and

Ras display reduced activity of mitochondrial respiratory complexes I and III

(*136*). These results are consistent with earlier work demonstrating direct

activation of mitochondrial complex I via SIRT3-mediated deacetylation (*69*).

Moreover, these findings suggest that there is impaired oxidative phosphorylation

and a heavier reliance on glycolytic metabolism in order to satisfy the energetic requirements of transformed $SIRT3^{-/-}$ fibroblasts, representing the primary hallmarks of the Warburg Effect. Coupling these findings with the knowledge that SIRT3 could potentially be regulating numerous mitochondrial proteins suggests that SIRT3 dysfunction may represent an important factor contributing to the poorly understood metabolic reprogramming that occurs during tumorigenesis (125). Consistent with this notion, SIRT3 is known to be downregulated in certain breast cancers (136). Furthermore, if SIRT3 can function as a tumor suppressor, it is logical to conceive that the putative mitochondrial acetyltransferase(s) counterbalancing the tumor-suppressive effects of SIRT3 would be characterized as oncogene(s). A multitude of mitochondrial proteins are acetylated in a reversible and nutrient- responsive manner. Accordingly, alterations in mitochondrial acetylation states, and hence, alterations in carbon substrate utilization, may contribute to the unusual preference for aerobic glycolysis and glutaminolysis often observed in numerous forms of cancer.

In addition to understanding the emerging importance of mitochondrial acetylation in age-associated diseases, it is also important to briefly discuss evidence for its involvement in the aging process in general. Heart failure and cancer are two pathophysiological states that display an exponentially increasing incidence with advancing age, and a role for the mitochondrial deacetylase SIRT3 in these respective conditions has been discussed (144). Complementing SIRT3's emerging role in diseases of aging is the interesting, though controversial observation that SIRT3 is the only member of the sirtuin family

reportedly linked to longevity in humans. An early study of the human *SIRT3* gene identified a guanine to thymine (G477T) single nucleotide polymorphism (SNP) located in exon three to be associated with survivorship in elderly males (*174*). Later, Bellizzi, *et al.* discovered a variable number of tandem repeat (VNTR) polymorphisms possessing enhancer activity at the *SIRT3* locus (*175*). The authors of this study further demonstrated that the inactive form of the *SIRT3* enhancer is underrepresented in a population exceeding 90 years of age, and thus concluded that the active VNTR enhancer is associated with longevity. Additional work has identified the activating elements that bind to this intronic VNTR enhancer sequence (*176*). In contrast to these studies, a meta-analysis of *SIRT3* SNP data coupled with a larger association study of the *SIRT3* chromosomal region in centenarians indicated no significant genomic variation that could be linked to longevity, with the exception of one possible SNP, rs939915 (*177*). Future analysis of genomic variation at the mammalian *SIRT3* locus, transgenic animals, and functional studies of the SIRT3 protein and its deacetylation targets will provide much-needed insight into the possible role this sirtuin plays in longevity and the aging process.

Summary and objectives

Although the acetylation of lysine residues has been known as a post-translational modification for over 40 years, it was not until the last 15 years that advances in technology and molecular techniques have enabled the rigorous study of this highly interesting regulatory mechanism. We now understand that

lysine acetylation rivals phosphorylation, in respect to both the sheer number of acetylated proteins in mammalian cells as well as in its regulatory capacity. Furthermore, global cellular acetylation states are largely dependent on mitochondrial-derived processes of acetyl-CoA formation. Many proteins within the mitochondria are now known to be reversibly acetylated and, in some cases, acetylation can regulate metabolism and the fate of carbon energy sources. Thus, reversible acetylation of mitochondrial proteins represents a new layer of protein regulation mediating acute and adaptive changes in mammalian metabolism. This new framework for understanding metabolism suggests that altered mitochondrial acetylation patterns can contribute to the pathological shifts in energy generation that are known to occur during heart failure, the rapid proliferation of malignant tumor cells, mitochondrial diseases, and indeed, the aging process in general.

Mitochondrial protein acetylation has not been studied in the context of any inherited disease, let alone one with mitochondrial and cardiac involvement. Therefore, the objective of the following study was to characterize the role of mitochondrial protein acetylation and metabolism in the pathogenesis of cardiomyopathy in the inherited mitochondrial disease Friedreich's Ataxia using conditional knockout mouse models. This study sought to 1) Determine if acetylation is altered in the Frataxin-deficient heart, 2) If so, understand the mechanism of altered acetylation, and 3) Understand alterations in Frataxin-deficient cardiac mitochondrial metabolism potentially related to protein acetylation.

CHAPTER II: FRIEDREICH'S ATAXIA REVEALS A MECHANISM FOR
COORDINATE REGULATION OF OXIDATIVE METABOLISM VIA FEEDBACK
INHIBITION OF THE SIRT3 DEACETYLASE[†]

Abstract

Friedreich's Ataxia (FRDA) is the most common inherited human ataxia
and is caused by a deficiency in the mitochondrial protein Frataxin. Clinically,
patients suffer progressive spinocerebellar degeneration, diabetes, and a fatal
cardiomyopathy, associated with mitochondrial respiratory defects. Recent
findings have shown that lysine acetylation regulates mitochondrial function and
intermediary metabolism. However, little is known about lysine acetylation in the
setting of pathological energy stress and mitochondrial dysfunction. We tested
the hypothesis that the respiratory chain defects in Frataxin deficiency alter
mitochondrial protein acetylation. Using two conditional mouse models of FRDA,
we demonstrate marked hyperacetylation of numerous cardiac mitochondrial
proteins. Importantly, this biochemical phenotype develops concurrently with
cardiac hypertrophy and is caused by inhibition of the NAD^+-dependent SIRT3
deacetylase. This inhibition is caused by an 85-fold decrease in mitochondrial
NAD^+/NADH and direct carbonyl group modification of SIRT3, and is reversed
with excess SIRT3 and NAD^+ *in vitro*. We further demonstrate that protein
hyperacetylation may be a common feature of mitochondrial disorders caused by
respiratory chain defects, notably, Cytochrome Oxidase I deficiency. These
findings suggest that SIRT3 inhibition and consequent protein hyperacetylation

represents a negative feedback mechanism limiting mitochondrial oxidative pathways when respiratory metabolism is compromised, and thus, may contribute to the lethal cardiomyopathy in FRDA.

Introduction

Friedreich's Ataxia (FRDA) is an autosomal recessive mitochondrial disorder caused by a homozygous triplet nucleotide repeat (GAA·TTC) expansion in intron 1 of the *FXN* gene located on chromosome 9q21.11 (*2*). This intronic expansion causes impaired transcription of the *FXN* gene and, consequently, a pathological deficiency of the *FXN* gene product, Frataxin (*5*). Frataxin is targeted to the mitochondrial matrix, where it is known to act as an iron-binding protein and participate in the proper assembly and function of iron-sulfur cluster (ISC) dependent proteins including complexes I, II, and III of the respiratory chain and aconitase of the tricarboxylic acid (TCA) cycle (*16, 18, 178*). Thus, Frataxin deficiency severely compromises both cellular respiration and overall mitochondrial function leading to energetic stress and ATP deficiency (*19*). Although patients develop multisystem disease including early spinocerebellar degeneration, ataxia, and diabetes, the primary cause of death is heart failure for nearly 85% of those afflicted (*179*). Similarly, although the phenotypes of the neuron-specific enolase (NSE) and muscle creatine kinase (MCK) – Cre conditional mouse models of FRDA differ, both models develop a fatal cardiomyopathy and impaired activity of iron-sulfur cluster-dependent respiratory complexes consistent with the human disease (*15*).

Recent work has demonstrated that lysine acetylation is a highly conserved and abundant post-translational modification within mitochondria that is responsive to nutrient availability and may contribute to the physiological adaptations of reduced caloric intake (*64, 120, 121, 123, 124, 180*). Multiple investigations have demonstrated a role for reversible mitochondrial enzyme deacetylation and, specifically, the NAD^+-dependent deacetylase SIRT3, in the regulation of fatty acid oxidation, the TCA cycle, electron transport via respiratory complexes I and II, and overall oxidative metabolism (*66, 69, 129, 181-183*). These studies suggest that mitochondrial protein acetylation could mediate metabolic changes In pathological states characterized by profound energetic stress and impaired cellular respiration, such as cardiomyopathies and inherited mitochondrial disorders, but this hypothesis remains unexplored (*184*). Using the above mentioned mouse models, we investigated whether acetylation states are altered in the setting of FRDA, and if so, sought to determine the mechanism.

Results

The heart contains the highest density of mitochondria of any organ in the mammalian body and heart failure represents the primary cause of death for nearly 85% of FRDA patients. Thus, we prepared whole heart lysates from WT, NSE, and MCK conditional mouse models of FRDA and performed Western blot analysis to assay protein acetyl-lysine modifications. Figures 2.1A & B show that heart lysates from both the NSE and MCK mouse models of FRDA exhibited
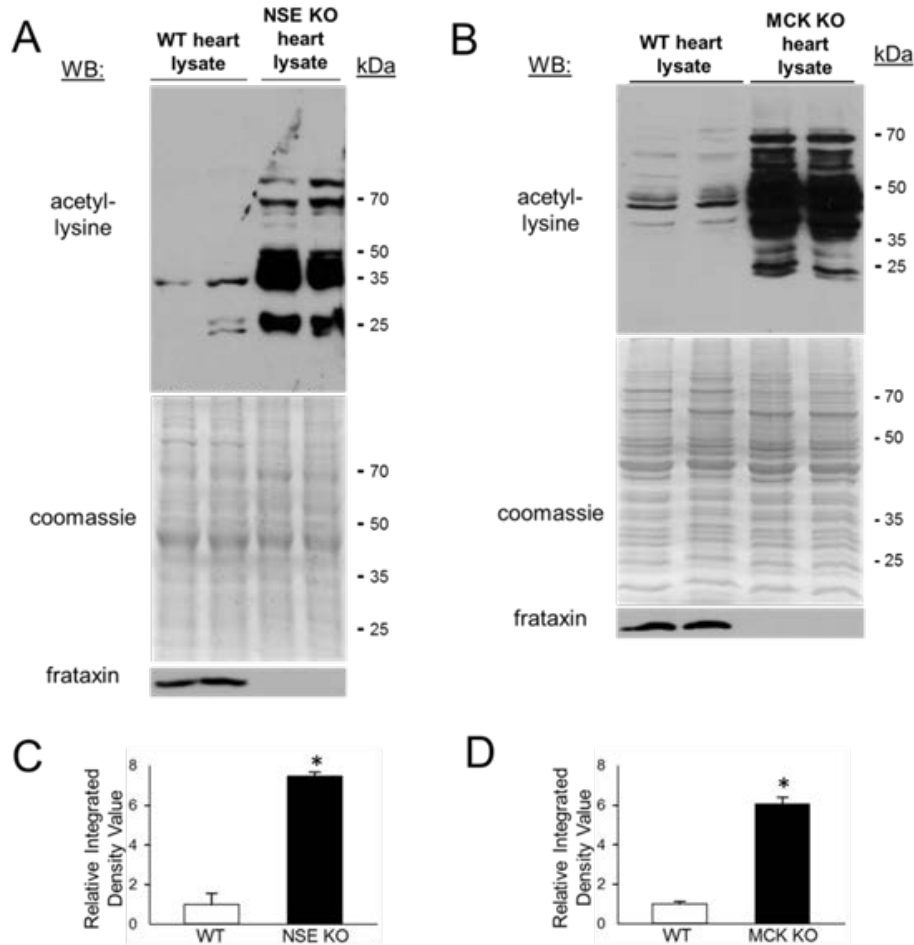
Figure 2.1 Frataxin-deficient hearts exhibit marked protein hyperacetylation
(*185*). (A) Western blot (WB) probing for internal acetyl-lysine residues using total
heart homogenates derived from 24 day-old wild-type (WT, n=2, lanes 1 and 2)
and 24 day-old NSE-Cre mouse models of FRDA (n=2, lanes 3 and 4). Below
this, the corresponding SDS-PAGE gel stained with Coomassie Blue and a
Western blot probing for Frataxin. (B) Similar to (A) only using total heart
homogenates prepared from 9 week-old WT (n=2, lanes 1 and 2) and 9 and 11
week-old MCK-Cre mouse models of FRDA (lanes 3 and 4, respectively). Below,
the corresponding gel stained with Coomassie Blue and a Western blot probing
for Frataxin. See also Figure S1. kDa: molecular weight in kilodaltons. (C) The
average relative integrated densitometry values from the acetyl-lysine Western
blot in (A) are shown (mean± SD; * *P*< .05). (D) The average relative integrated
densitometry values from the acetyl-lysine Western blot in (B) are shown (mean±
SD; * *P*< .05).

marked increases in acetyl-lysine modifications as compared to age-matched

control hearts, and these are significant (Figure 2.1C & D). The differences were

most dramatic in proteins with an estimated molecular weight between

approximately 30 and 75 kDa. In contrast to heart tissue, whole brain lysates

from the NSE models and skeletal muscle lysates from the MCK models showed

no change in acetylation state when compared to WT control lysates (Figure 2.2).

Figure 2.2 NSE brain and MCK skeletal muscle do not show altered acetylation. NSE mouse model whole brain lysate and MCK mouse model whole skeletal muscle lysate exhibit no change in acetylation state when compared to age-matched WT control tissues.

Cardiac cellular sub-fractionation of heart samples to determine the sub-

cellular distribution of hyperacetylated proteins. Analysis of the purity of these
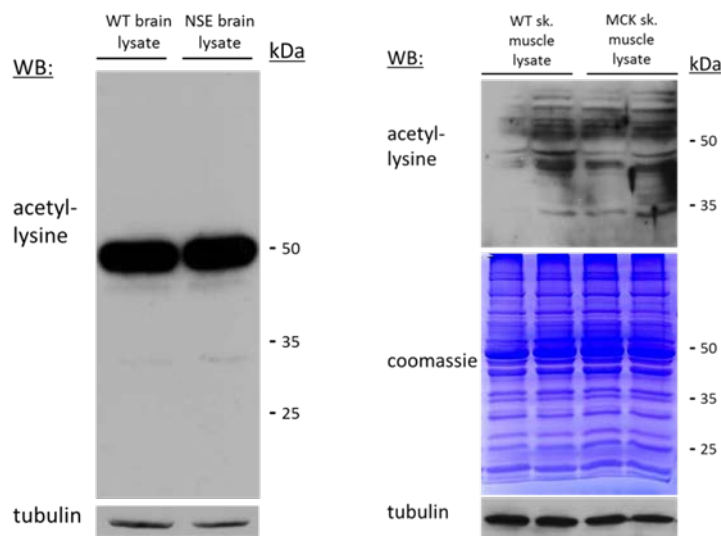
mitochondrial preparations showed that nuclear and cytosolic proteins were excluded and they were highly enriched for markers of both the outer and inner mitochondrial membranes (Figure 2.3A). Using day 24 wild-type (WT, n=2) control and NSE-Cre Frataxin-deficient cardiac mitochondrial preparations (n=2), we performed Western blot analysis for acetyl-lysine modifications. Control cardiac mitochondria exhibited several acetylated proteins detectable by Western blot which is consistent with prior findings (*123*). However, Frataxin-deficient cardiac mitochondria displayed marked hyperacetylation of numerous proteins (Figure 2.3B and Figure 2.4). This was accompanied by a characteristic downregulation of respiratory complex I and II (succinate dehydrogenase) that was present as early as 7 days post-natal (Figure 2.5). Levels of the dominant mitochondria-localized NAD^+-dependent deacetylase SIRT3 displayed a mild, though insignificant, increase in Frataxin-deficient mitochondrial preparations (Figure 2.3B and C), which is consistent with prior work demonstrating induction of SIRT3 in cultured cardiomyocytes subjected to various forms of stress (*104*). These results indicated that the hyperacetylation observed at the level of whole cardiac lysate is predominantly localized to mitochondria.

Because the NSE-Cre mouse models of FRDA only begin to develop cardiac hypertrophy in their second week of life (*15*), we next sought to examine the developmental profile of mitochondrial protein acetylation. We prepared cardiac mitochondria for Western analysis from WT control and NSE-Cre mice at post-natal days 7, 17, and 24. At post-natal day 7, NSE-Cre cardiac mitochondrial proteins exhibit only a mildly increased acetylation state as

compared to their wild-type counterparts (Figure 2.3D and 2.5). However, at post-natal day 17, NSE-Cre cardiac mitochondria display increased acetylation of cardiac mitochondrial proteins as compared to their WT counterparts and this difference becomes more dramatic by post-natal day 24. Importantly, the progressive increase in cardiac mitochondrial protein acetylation over this time frame corresponded with the development of cardiac hypertrophy in the NSE-Cre models (Figure 2.3E).

Prior work had demonstrated that genetic ablation of the deacetylase SIRT3, but not SIRT4 or SIRT5, caused hyperacetylation of mitochondrial proteins in liver and brown adipose tissue (*186*). These findings immediately suggested that hyperacetylation in Frataxin deficiency could be caused by impairment or inhibition of SIRT3. Respiratory chain dysfunction is known to alter the cellular and mitochondrial redox state (NAD^+/NADH) via impaired oxidation and consequent accumulation of NADH generated by the TCA cycle (*187-189*). Importantly, sirtuin activity is responsive to redox state (*190*), although this finding is somewhat controversial (*191*). Thus, we determined the redox state of WT and Frataxin-deficient cardiac mitochondria preparations. WT heart mitochondria displayed robust NAD^+ levels and over 100-fold less NADH by comparison, which is consistent with highly oxidative cardiac catabolism and a continuous demand for carbon fuels (Figure 2.6A). Frataxin-deficient mitochondria displayed a mild, though insignificant, increase in NAD^+ levels when compared to WT. In striking contrast, Frataxin-deficient mitochondrial NADH

levels were, on average, over 95-fold greater than in WT mitochondria ($P<.005$)

resulting in a corresponding NAD^+/NADH ratio that was 85-fold less than in WT
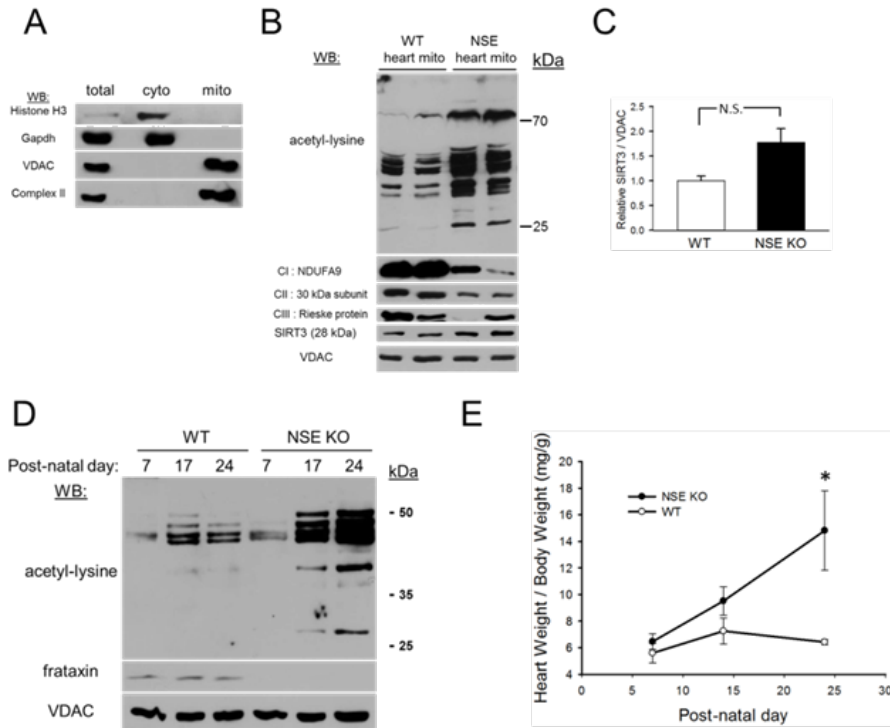
Figure 2.3 Hyperacetylation in Frataxin-deficient hearts is localized to mitochondria and develops progressively with cardiac hypertrophy (*185*). (A) Western blot (WB) demonstrating the purity of the cardiac mitochondrial preparations. Histone H3 was used as a nuclear marker, GAPDH as a cytoplasmic marker, VDAC as a mitochondrial outer membrane marker, and Complex II as a mitochondrial inner membrane marker. (B) Hyperacetylation is localized to cardiac mitochondria. Western blot probing for internal acetyl-lysine residues using 25 µg of isolated mitochondrial protein derived from 2-3 pooled hearts from 24 day-old wild-type (WT, n=2, Lanes 1 and 2) hearts and 24 day-old NSE Frataxin KO hearts (n=2, Lanes 3 and 4). Below, a series of Western blots probing for the respiratory Complex I subunit NDUFA9, the Complex II 30 kilodalton iron-sulfur subunit, the Complex III Rieske protein, and the NAD^+-dependent deacetylase SIRT3. The mitochondrial outer membrane protein voltage-dependent anion channel (VDAC) was used as a loading control. See also Figure 3.4. kDa: molecular weight in kilodaltons. (C) The calculated densitometry for SIRT3 relative to the loading control VDAC shown in Figure 2B (mean ± SD; N.S.: not significant). (D) Hyperacetylation develops progressively. Western blot probing for internal acetyl-lysine residues in 25ug of mitochondrial protein preparations isolated from 2-3 WT or NSE-Cre Frataxin knockout hearts

at day 7, day 17, and day 24 in their post-natal development. Below, a Western blot probing for Frataxin and VDAC was used as a loading control. Note: Due to very small amounts of heart material and inefficient mitochondrial isolation at post-natal Day 7, mitochondrial protein from day 7 WT and NSE animals was overloaded by 18.2% and 62%, respectively, with respect to the other samples to achieve normalization of mitochondrial protein. See also Figure S2. kDa; molecular weight in kilodaltons. (E) Cardiac hypertrophy develops concurrently with protein hyperacetylation. A graph assessing the post-natal development of cardiac hypertrophy in the NSE KO mice as measured by heart weight divided by body weight (mean ±SD; n= 3 measurements at each time point; *P<.05).

animals (Figure 2.6A inset). The observed accumulation of NADH and

consequent shift in mitochondrial redox state is consistent with the impairments

of mitochondrial respiration found in both human FRDA patients, and the NSE

and MCK animal models (*15, 18*).

An increased sensitivity to oxidative stress is believed to contribute to the

pathogenesis of FRDA (*192*). A proteomics investigation of the MCK mouse

heart revealed upregulation of the antioxidant enzymes glutathione *S*-transferase

and DJ-1 (*193*). Furthermore, FRDA patients were shown to have increases in

plasma levels of malondialdehyde, a product of lipid peroxidation (*60*). A recent

report demonstrated that SIRT3 can be directly modified and allosterically

inhibited by sub-physiological levels of 4-hydroxy-2-nonenal (4-HNE), an

endogenous product of lipid peroxidation (*194*). Therefore, we investigated if

SIRT3 is differentially modified by 4-HNE in Frataxin-deficient cardiac

mitochondria. We pulled down all reactive aldehyde containing mitochondrial

proteins following their derivatization to biotin hydrazide, and then performed

Western blot analysis for SIRT3. SIRT3 in WT heart mitochondria exhibited a

small amount of 4-HNE modification. In contrast, SIRT3 in Frataxin-deficient

mitochondria exhibited a marked increase in 4-HNE modification, (Figure 2.6B)

suggesting that SIRT3 may be directly inhibited via carbonyl group adduction in

the setting of Frataxin deficiency. We next analyzed the acetylation states of
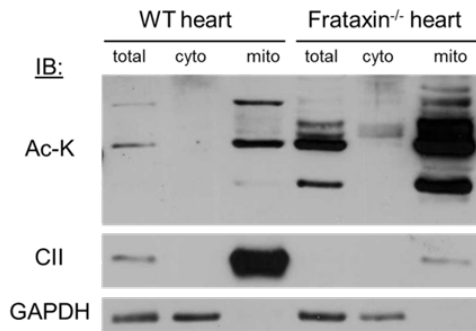
Figure 2.4 Protein hyperacetylation in Frataxin-deficient hearts is localized to mitochondria (*185*). Western blot on 20 µg of total heart homogenate, cytoplasmic fraction, and mitochondrial fraction probing for internal acetyl-lysine residues. Below, Western blots probing for the mitochondrial marker complex II iron-sulfur subunit and the cytoplasmic marker GAPDH.

known targets of SIRT3-mediated deacetylation. The Complex I subunit NDUFA9

was previously found to be a specific target of SIRT3-mediated deacetylation

(*69*). To test the hypothesis that SIRT3 is inhibited in Frataxin-deficient hearts,

we immunoprecipitated mitochondrial acetyl-lysine proteins followed by Western

blot analysis for NDUFA9. Consistent with previous findings, we observed a

minimal amount of NDUFA9 acetyl-lysine signal in the WT mitochondrial

immunoprecipitate. However, the Frataxin-deficient mitochondrial

immunoprecipitate displayed a greater acetylated NDUFA9 signal despite

downregulation of NDUFA9 as seen in the input (Figure 2.6C). We additionally

analyzed the acetylation state of a second specific target of SIRT3-mediated

deacetylation, acetyl-CoA synthetase 2 (AceCS2) (*120, 121*). Similarly, this

analysis revealed a greater amount of acetylated AceCS2 in the Frataxin-

deficient condition as compared to the WT (Figure 2.6D). The increased

acetylation states of two known targets of SIRT3-mediated deacetylation

indirectly demonstrated that SIRT3 is inhibited in Frataxin-deficient cardiac

mitochondria. Importantly, increases in the acetylation states of NDUFA9 and
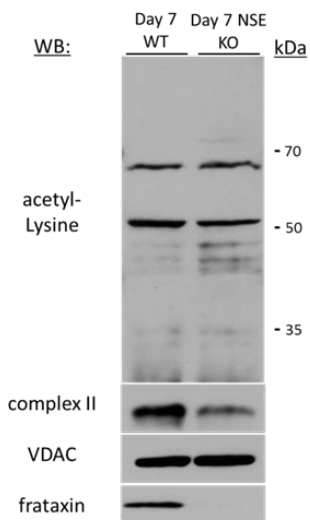
Figure 2.5 Frataxin-deficient cardiac mitochondrial proteins display increases in acetylation as early as post-natal day 7. They also exhibit downregulation of the respiratory complex II iron-sulfur subunit (*185*). VDAC was used as a loading control.

AceCS2 are linked to a decrease in the activity of respiratory complex I and the

synthesis of activated acetate, respectively (*69, 120*).

Prior studies have demonstrated that SIRT3 exhibits acetyl-lysine target

specificity and does not deacetylate all acetyl-lysines present on a target protein

(*66, 195*). Based on these findings, we reasoned that if hyperacetylation of

Frataxin$^{-/-}$ mitochondrial protein lysine residues were caused by an inhibition of

endogenous SIRT3, then incubating with excess SIRT3 and NAD$^+$ *in vitro* would

restore SIRT3 activity resulting in deacetylation of its specific acetyl-lysine targets. Accordingly, we incubated solubilized WT and Frataxin-deficient cardiac mitochondrial proteins with glutathione *S*-transferase tagged, processed human recombinant SIRT3 (GST-hSIRT3) in the presence or absence of NAD^+ to assay for changes in acetylation states. Incubating solubilized WT or Frataxin$^{-/-}$ cardiac mitochondrial homogenates with NAD^+ alone caused no change in acetylation signal. In contrast, incubating Frataxin-deficient mitochondrial protein with 3 µg of GST-hSIRT3 and NAD^+ resulted in a marked reduction of acetyl-lysine signal from nearly every protein band, while completely eliminating the acetyl-lysine signal of multiple bands (Figure 2.6E). Furthermore, the observed reduction in acetylation signal was abolished upon withdrawal of NAD^+ from the incubation buffer, demonstrating that the hyperacetylated protein lysine residues in Frataxin-deficient cardiac mitochondria are, indeed, specifically sensitive to NAD^+-dependent SIRT3-mediated deacetylation and that the observed hyperacetylation is not caused by a general increase in non-specific lysine acetylation. Taken together, these data strongly suggest that hyperacetylation in Frataxin-deficient mitochondria is due to both redox-state and lipid-peroxidation-mediated inhibition of endogenous SIRT3.

We obtained cardiac mitochondria from SIRT3 KO animals on a C57BL/J6 genetic background to compare acetylation profiles with our Frataxin-deficient cardiac mitochondria. In spite of the differences between the two animal models including genetic background, age, and one a model of disease and the other a simple gene ablation, comparing the acetylation state of Frataxin-deficient

cardiac mitochondria to SIRT3-deficient cardiac mitochondria yielded many

similarities. Both models exhibited hyperacetylation of mitochondrial proteins and

many hyperacetylated protein bands appeared at identical molecular weights

(Figure 2.7). The Frataxin-deficient cardiac mitochondria displayed an overall
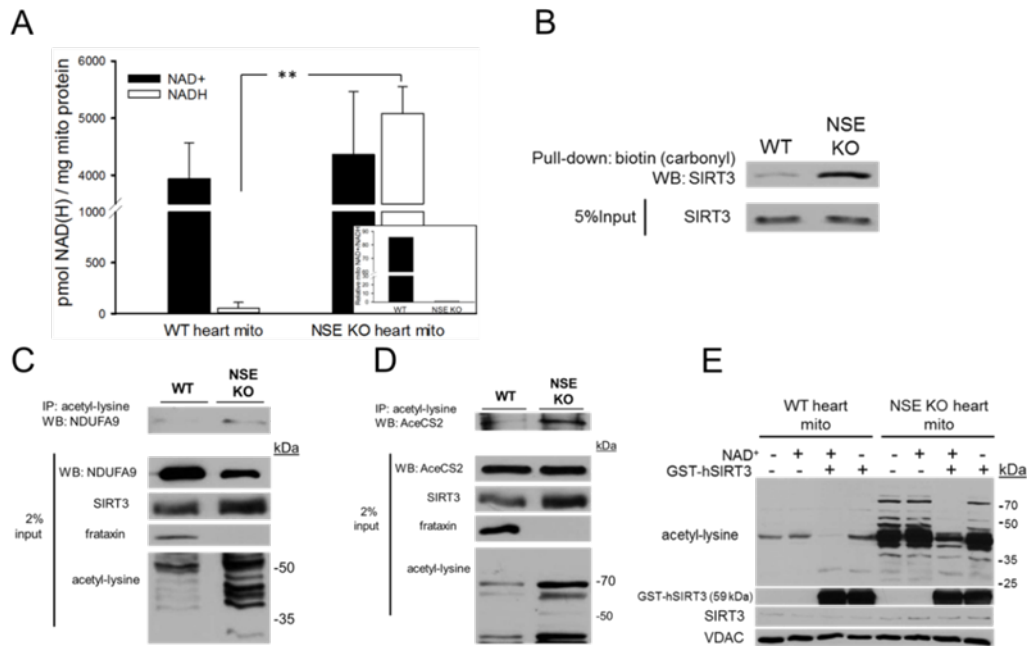
Figure 2.6 Hyperacetylation in Frataxin-deficient hearts may be caused by impaired SIRT3 activity (*185*). (A) Determination of nicotinamide adenine dinucleotide (NAD$^+$) and reduced NAD$^+$ (NADH) levels in WT and Frataxin-deficient cardiac mitochondrial preparations. Individual measurements were performed on a pooled sample of at least 300 μg of fresh cardiac mitochondria derived from 2-3 hearts and normalized to total mitochondrial protein input (n=3-5 biological replicates per condition, mean± SD; ** *P*<.005). Figure inset: the ratio of NAD$^+$ to NADH for WT and NSE-KO heart mitochondria. (B) SIRT3 is differentially modified by a carbonyl group in Frataxin-deficient mitochondria. This modification was previously shown to allosterically inhibit SIRT3 activity (*194*). The shown figure is representative of two independent experiments. (C) Targets of SIRT3-mediated deacetylation are hyperacetylated in Frataxin-deficient hearts. Immunoprecipitation of 500ug WT and NSE-Cre cardiac mitochondrial proteins with an acetyl-lysine antibody and subsequent Western blot probing for a known target of SIRT3-mediated deacetylation, the respiratory complex I subunit NDUFA9. Below, Western blots of 2 percent of the total mitochondrial lysate input used for the immunoprecipitations showing NDUFA9, SIRT3, Frataxin, and the acetylation states of both samples. Cardiac mitochondrial preparations were derived from 2-3 pooled hearts per condition. The figure shown is representative of three independent experiments. (D) Similar to (C), only examining acetyl-CoA

synthetase 2 (AceCS2) as a target of SIRT3-mediated deacetylation. (E) Recombinant SIRT3 in the presence of 10mM NAD$^+$ reverses mitochondrial protein hyperacetylation in Frataxin-deficient hearts. Below are Western blots showing recombinant SIRT3, endogenous SIRT3, and VDAC which was used as a loading control. kDa; molecular weight in kilodaltons.
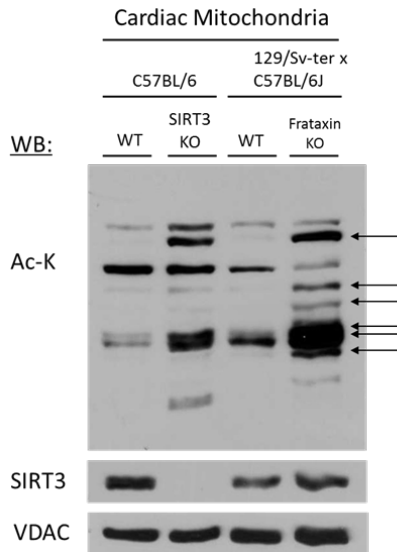
Figure 2.7 Comparing the cardiac mitochondrial protein acetylation profiles between SIRT3 KO and Frataxin KO animals. The arrows indicate a protein band which is hyperacetylated in both the SIRT3 KO and Frataxin KO conditions. Below, a Western Blot probing for SIRT3 and VDAC as a mitochondrial loading control.

increase in acetylation intensity as compared to the SIRT3-deficient cardiac

mitochondria. This could suggest that a portion of the acetylation observed in

Frataxin-deficient mitochondria occurs independently of SIRT3 inhibition.

Alternatively, this finding could reflect differences in the abundance of acetylated

proteins between substantially different animal models. Nonetheless, these

results further support the findings that hyperacetylation of mitochondrial proteins

in the Frataxin-deficient heart is, in part, caused by a loss of SIRT3 deacetylase

activity.

Friedreich's Ataxia is a mitochondrial disorder that has a distinctive cause and unique pathogenesis. However, the observed accumulation of NADH in Frataxin-deficient mitochondria and consequent drop in NAD^+/NADH ratio is often a common biochemical feature of respiratory chain disorders, regardless of mutational origin (*38, 189, 196*). Taking this common feature into consideration with the findings that SIRT3 deacetylase activity is NAD^+-dependent and likely to be responsive to this change in mitochondrial NAD^+/NADH balance, we hypothesized that other mitochondrial disorders caused by respiratory chain defects would display alterations in cardiac acetylation profiles. To test this, we obtained whole cardiac lysates from the cytochrome oxidase I (COI) mouse model, which harbors a mitochondrial DNA mutation causing a defect of respiratory complex IV, and the adenine nucleotide translocase knockout (ANT1$^{-/-}$) mouse model, which models a non-respiratory chain defect with a deficiency of ATP translocation (*197, 198*). These mice model the human mitochondrial disorders autosomal dominant progressive external ophthalmoplegia (adPEO), and Leber's Hereditary Optic Neuropathy (LHON), respectively, and both models develop cardiomyopathy (*199, 200*).

Analyzing cardiac acetylation via Western blot revealed mild to moderate increases in acetylation in 12 month old COI hearts as compared to 13 month-old control hearts (Figure 2.8A). Interestingly, 20 month old ANT1$^{-/-}$ hearts exhibited a small decrease in acetyl-lysine profiles when compared to 18 month old control hearts (Figure 2.8B). Taken together with our data from the Frataxin-deficient

mice, these results suggest that increases in protein acetylation may be a common feature of respiratory chain malfunction in the mammalian heart.
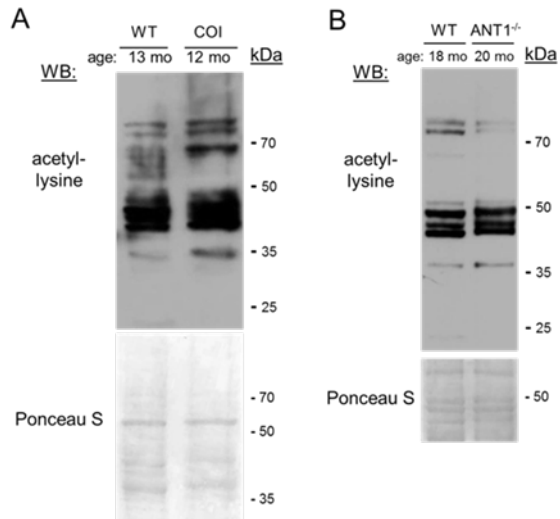
Figure 2.8 Respiratory chain defects may cause protein hyperacetylation (*185*). (A) Western blot probing for internal acetyl-lysine residues using 25 µg of whole heart lysate derived from a 13 month-old wild-type (WT) animal and a 12 month-old animal harboring a missense mutation in the mitochondrial genome-encoded protein subunit of the respiratory chain enzyme cytochrome *c* oxidase (COI). Below, the same membrane used for Western blotting above stained with the non-specific protein marker Ponceau S. (B) Western blot probing for internal acetyl-lysine residues using 25 µg of whole heart lysate prepared from a 18 month-old WT animal and a 20 month-old animal lacking the non-respiratory chain mitochondrial inner membrane protein adenine nucleotide translocase 1 (ANT1^{-/-}). Below, the same membrane used for Western blotting above stained with Ponceau S. (C) The calculated integrated densitometry values for acetyl-lysine signal relative to Ponceau S signal from Figure 4A. (D) Similar to C, only calculated from Figure 4B. kDa; molecular weight in kilodaltons.

Discussion

In this study, it was demonstrated that the respiratory chain defects accompanying Frataxin deficiency cause progressive hyperacetylation of cardiac mitochondrial proteins due to the inhibition of a major positive regulator of oxidative metabolism, the SIRT3 deacetylase (*183, 201*). These findings present

a novel disease phenotype and reveal a negative feedback mechanism that may serve to coordinately suppress mitochondrial oxidative pathways, and that may be hyperactive in the setting of respiratory chain malfunction.

Defects in cellular respiration can be inherited as mitochondrial disease, or acquired over a lifetime via somatic mutations, and are linked to many conditions including neurodegenerative disease, diabetes, heart failure, cancer and indeed, the aging process in general (*196*). The involvement of cellular respiration in numerous common human pathologies emphasizes the need for greater understanding of the pathophysiological processes that occur in response to respiratory chain compromise. SIRT3-mediated deacetylation has recently emerged as a major mechanism regulating the activity of mitochondrial oxidative and intermediary metabolism. SIRT3 is also uniquely poised to respond to the flux of mitochondrial NAD^+ and NADH, which is determined, in large part, by the capacity of the respiratory chain to oxidize NADH. This capacity is severely decreased in FRDA, as well as in other mitochondrial defects such as cytochrome c oxidase (complex IV) deficiency (*187*), causing an accumulation of NADH and, consequently, a redox state of perceived nutrient excess. In this setting, it is logical to predict that mitochondria would have a mechanism to restrain oxidative metabolism and the further generation of NADH, as well as spare the cell from damaging ROS that would be produced by forcing electrons through a dysfunctional respiratory chain. Indeed, it has been known for several decades that a decrease in NAD^+/NADH redox balance has a largely inhibitory effect on many enzymes of intermediary mitochondrial metabolism (*202*). Based

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on these observations and predictions, our data suggests that this mechanism

involves inhibition of the NAD^+-dependent SIRT3 deacetylase mediated through

a dramatic decrease in mitochondrial redox-state, as measured by the

NAD^+/NADH ratio, and direct modification by lipid peroxidation.

Recent studies have shown that SIRT3-mediated deacetylation activates

the respiratory chain via Complexes I and II, fatty acid oxidation via LCAD, the

TCA cycle via isocitrate dehydrogenase, and the production of acetyl-CoA via

AceCS2, among others (*66, 69, 120, 129*). Additionally, SIRT3 deficient

fibroblasts show a decrease of carbon intermediates in the TCA cycle (*203*).

Together, these studies indicate a key role for SIRT3 in the maintenance of

oxidative metabolism (*183*) and may be especially important in the heart, which

derives as much as 90% of its ATP from oxidative phosphorylation (*35*). Indeed,

SIRT3 deficient mice exhibit reduced cardiac ATP levels, are hypersensitive to

cardiac stress, and develop an early onset cardiac hypertrophy (*69, 138, 204*).

These findings suggest that SIRT3 inhibition, in addition to impaired ISC

biosynthesis, may contribute to FRDA cardiomyopathy by further limiting key

components of oxidative and intermediary metabolism, such as the respiratory

chain, the TCA cycle, and the β-oxidation of fatty acids. In addition to its

metabolic functions, SIRT3 is known to directly activate antioxidant defense

mechanisms via deacetylation of mitochondrial MnSOD and isocitrate

dehydrogenase of the glutathione system (*182, 205, 206*). These important

findings suggest that inhibition of SIRT3 in FRDA could underlie the increased

sensitivity to oxidative stress (*192*). Thus, it is logical to predict that Frataxin

deficiency promotes a vicious cycle whereby improper assembly of ISC-dependent respiratory complexes and their consequently reduced activity causes SIRT3 inhibition, leading to the accumulation of protein lysine acetylation which further inhibits the activity of the respiratory chain and oxidative metabolism (Figure 2.9). This model would be consistent with the progressive increase in mitochondrial protein acetylation we observe during the post-natal development of the NSE-Cre Frataxin$^{-/-}$ mice (Figure 2.3D). Interestingly, a recent report noted that Frataxin-deficient yeast show decreased acetylation of mitochondrial proteins (*207*), which may reflect the yeast preference for glucose as a carbon source instead of fatty acids utilized by heart mitochondria, or that no sirtuins are known to function within yeast mitochondria. The extent to which SIRT3 inhibition and mitochondrial protein hyperacetylation influence these diverse processes and the pathogenesis of FRDA cardiomyopathy is likely to be very important and remains to be explored.
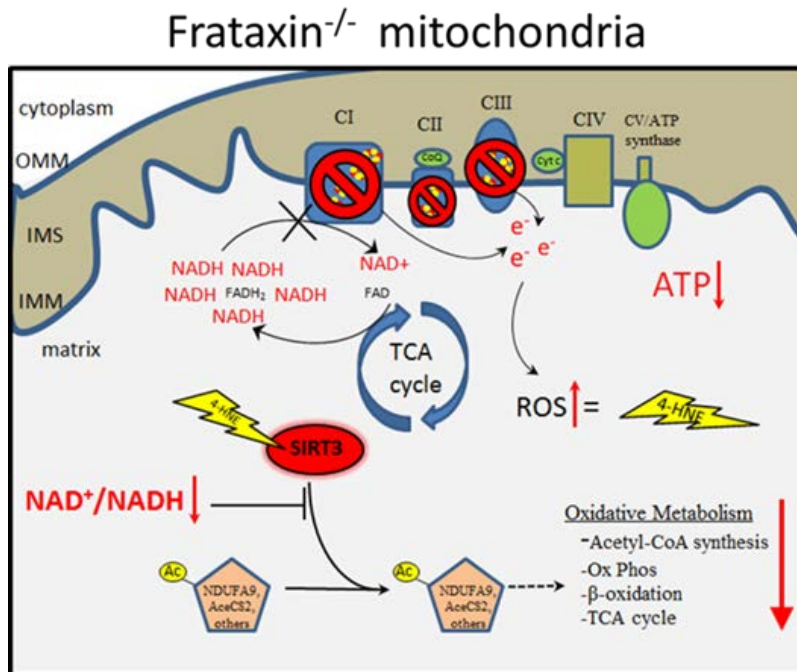
Figure 2.9 Cartoon schematic summarizing the proposed mechanism of SIRT3 inhibition in Frataxin-deficient cardiac mitochondria (*185*). Impaired ISC biosynthesis in Frataxin$^{-/-}$ mitochondria results in defects of respiratory complexes I, II, and III, which cause impaired oxidation and accumulation of NADH generated by the TCA cycle. The resulting dramatic decrease in the mitochondrial NAD$^+$/NADH ratio (redox state) creates a reducing environment mimicking nutrient excess. In addition, an increased sensitivity to oxidative stress consequently promotes lipid peroxidation and the formation of 4-HNE protein carbonyl adducts. The dramatic decrease in mitochondrial redox state and increase in 4-HNE modified SIRT3 work in concert to inhibit SIRT3 activity, causing protein hyperacetylation and most likely reduced activity of SIRT3 target proteins which have been shown to play key roles in the maintenance of oxidative and intermediary metabolism. OMM: outer mitochondrial membrane, IMS: intermembrane space, IMM: inner mitochondrial membrane, ROS: reactive oxygen species, 4-HNE: 4-hydroxy-2-nonenal, NDUFA9: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, AceCS2: acetyl-CoA synthetase 2.

Materials & Methods

Animal use statement. The use of laboratory mice in this study was approved by the Indiana University School of Medicine's Institutional Animal Care and Use Committee (IACUC) and Laboratory Animal Resource Center (LARC). All conducted experiments conform to the American Veterinary Medical Association's Panel on Euthanasia.

Frataxin conditional knockout mouse breeding and genotyping. Mice homozygous for a floxed exon 4 of the *Frda* gene (*Frda*^L3/L3^) were crossed with mice heterozygous for a deletion of *Frda* exon 4 (*Frda*^+/Δ^) carrying a tissue-specific Cre transgene driven by the neuron-specific enolase (NSE), or muscle creatine kinase (MCK), promoters to generate a mouse with *Frda* exon 4 deletion restricted primarily to the heart and brain or heart and skeletal muscle, respectively (*15*). The primers used to detect the presence of exon 4 for genotyping were 5'-CTGTTTACCATGGCTGAGATCTC-3' and 5'-CCAAGGATATAACAGACACCATT-3' which generates a 500 bp product. The primers used to detect the presence of the Cre transgene were 5'-CCGGTCGATGCAACGAGTGAT-3' and 5'-CAAAGAGTCAT CCTTAGCGCC-3', which generate a 790 bp band. Mice that are genotyped as Frda^L3/Δ^, Cre+ represent the conditional knockout animals.

Mouse tissue preparation. For the isolation of whole heart, brain, skeletal muscle, or liver homogenate, animals were sacrificed by CO_2 asphyxiation and the tissue was immediately excised, washed and minced in cold phosphate-buffered saline. The tissue was then placed in 1-2 ml of ice-cold complete RIPA

buffer containing an EDTA-free protease inhibitor cocktail (Roche product #05056489001), and 10 mM nicotinamide (NAM), 200 nM Trichostatin A, and 5 mM sodium butyrate as deacetylase inhibitors. The tissues were then homogenized with at least 10 passes of a motor-driven Potter-Elvehjem homogenizer at high speed. The whole tissue lysates were then transferred to 1.5 ml tubes and spun at 17,000 x g for 10 min at 4°C in a desktop centrifuge to pellet membranes and tissue debris. The supernatant was saved and immediately frozen on dry-ice for later analysis. Frozen, RIPA buffer solubilized ANT1 $^{-/-}$ and COI missense mutation cardiac lysates were a generous gift of Dr. Douglas C. Wallace.

Isolation of Cardiac Mitochondria. Wild-type or Frataxin conditional KO mice were sacrificed by CO_2 asphyxiation and their hearts were immediately removed and submerged in ice-cold mitochondrial isolation buffer (MIB) containing 220mM mannitol, 70 mM sucrose, 30 mM Tris-Cl (pH 7.4), 0.5 mM EGTA, and 0.1% BSA. Heart tissue was minced and washed in MIB to eliminate blood and serum contaminants, then quickly weighed. Minced heart tissue from 2-3 animals was submerged 3 ml/g MIB containing an EDTA-free protease inhibitor cocktail (Roche) and 10 mM nicotinamide (NAM), 200 nM Trichostatin A, and 5 mM sodium butyrate as deacetylase inhibitors. Heart tissue was homogenized with four passes of a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle at medium speed on ice. An additional 7 ml/g MIB was added to the heart homogenate, which was then transferred to a pre-chilled 50 ml conical tube. A crude mitochondrial pellet was obtained via a standard differential centrifugation

method. Briefly, nuclei and the heavy cell fraction are removed via centrifugation for 15 minutes at 1000 x g at 4°C. The resulting post-nuclear supernatant was subjected to centrifugation for 15 min at 10,000 x g to pellet mitochondria. The supernatant was removed as the cytosolic fraction and sedimented mitochondria were gently resuspended in 1 ml ice-cold MIB containing deacetylase inhibitors and then diluted with MIB to a total volume of 10ml and centrifuged again for 15 min at 10,000 x g. The supernatant was then removed and sedimented mitochondria are gently washed in 0.5 M KCl to remove endoplasmic reticulum and lysosome contaminants as described previously (*208*). When the downstream application was Western blotting, the sedimented mitochondria were solubilized in complete RIPA buffer containing protease and deacetylase inhibitors (see Mouse tissue preparation) and frozen until later analysis. For immunoprecipitations, the pelleted mitochondria were resuspended in ice-cold buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, and an EDTA-free protease inhibitor tablet and immediately frozen at -80°C. For NAD$^+$ and NADH measurements, sedimented mitochondria were immediately resuspended in 180 µl of ice-cold 1mM Tris-Cl pH 7.4, 150 mM NaCl and extracted appropriately (see Mitochondrial NAD$^+$ and NADH measurements).

Antibodies and Western Blotting. Antibodies used in this study include anti-acetyl-lysine and acetyl-lysine antibody-conjugated agarose beads (Immunechem), anti-acetyl-lysine (Cell Signaling), anti-complex II 30 kDa subunit, anti-NDUFA9 and anti-aconitase (Mitosciences), anti-SIRT3 (gift of E.Verdin, Gladstone Institute, UCSF (*66*)), anti-Frataxin (gift of G. Isaya, Mayo

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Clinic), anti-SIRT3 D22A3, anti-VDAC D73D12, and anti-histone H3 (Cell Signaling), anti-Gapdh and anti-tubulin (Sigma), and anti-AceCS2 (ACSS1)(Abcam). Following transfer of the SDS-PAGE gel proteins to nitrocellulose membranes, the membrane was blocked for 30 min in PBS-0.05% Tween-20 supplemented with 5% non-fat dry milk. Signals were visualized with SuperSignal West chemiluminescent substrate (Thermo-Pierce). Western blot signal intensities were determined by density quantification using ImageJ software where appropriate. Protein concentrations were determined using the BCA method (Pierce).

Immunoprecipitation of acetyl-lysine proteins. For immunoprecipitation of acetyl-lysine proteins and detection of NDUFA9 and AceCS2 acetylation, 500ug to 1mg of cardiac mitochondria was solubilized for 30 min on ice in 1% lauryl maltoside (Mitosciences) and centrifuged at 16,000 x g for 10min at 4°C to pellet debris and lipid membrane components. Anti-acetyl-lysine antibody (Cell Signaling) was loaded with the solubilized mitochondrial supernatant at a ratio of 1:25 w/w and incubated overnight at 4°C with gentle agitation. Following incubation with the anti-acetyl-lysine antibody, the sample was incubated at 4°C with gentle agitation for an additional 2 hours with 7.5 µl Protein A agarose (Invitrogen) plus 7.5 µl Protein G agarose (Roche). Agarose was collected via a 1 min centrifugation at 400 x g at 4°C on a desktop centrifuge and the unbound mitochondrial supernatant was carefully removed from above the agarose beads. The beads were washed 3 times for 5 min in 500 µl IP buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EGTA, and 0.5% NP-40 at 4°C and then

incubated in 30 µl SDS sample loading buffer and boiled at 95°C for 5 min. The immunoprecipitated sample was then resolved on a 12% SDS-PAGE and processed for Western blotting. These methods were adapted from Ahn, *et al.* (*69*).

Mitochondrial NAD$^+$ and NADH measurements. NAM was excluded from MIB used to isolate mitochondria for NAD$^+$ measurements as the enzyme nicotinamide phosphoribosyltransferase (Nampt) catalyzes the formation of NMN, an NAD$^+$ precursor, from NAM. NAD(H) measurements were performed with a cycling assay according to the manufacturer's instructions (Bioassay Systems). Briefly, NAD$^+$ is extracted in the mitochondrial sample via addition of strong acid and then heated at 60°C to eliminate the presence of the reduced nucleotide (NADH). NADH is extracted in mitochondrial sample homogenate via addition of strong base and then heated at 60°C to eliminate the presence of the oxidized nucleotide (NAD$^+$). The extractions are neutralized with the opposite extraction buffer and the dehydrogenase cycling assay is performed in which, in the presence of lactate, lactate dehydrogenase, diaphorase and MTT formazan reagent, the concentration of NAD$^+$ or NADH present in the sample is proportional to the amount of reduced MTT formation which is colorimetrically detected with a plate reader at 562 nm (*209, 210*). Sample concentrations are determined via an NAD$^+$ standard curve. The coenzyme concentration was normalized to the protein concentration of the sample.

***In vitro* SIRT3 deacetylation assay.** Two hundred microliters of 2 µg/ul Frataxin$^{-/-}$ cardiac mitochondria frozen in 50mM Tris-Cl pH 7.4, 150mM NaCl were thawed

on ice and solubilized in 1% lauryl maltoside for 30 min on ice and centrifuged at 16,000 x g for 10min at 4°C to pellet debris and lipid membrane components. 100 µl of the soluble mitochondrial supernatant was mixed into 100 µl of 2x deacetylase buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, with or without 20 mM NAD^+). Recombinant GST-tagged SIRT3 corresponding to amino acids 101-399 of human SIRT3 (Sigma) in 40 mM Tris-Cl pH 8.0, 240 mM NaCl, and 20% glycerol was added to 30 µl (30 µg) of the 1:1 mitochondrial protein : 2x deacetylase buffer mix in a 1.5ml tube. An appropriate amount of buffer containing only 40 mM Tris-Cl, 240 mM NaCl, and 20% glycerol was added to incubations lacking SIRT3 or less than 3 µg of SIRT3 to equalize the incubation volumes. The reactions were incubated for 4 hours at 37°C at 400rpm in an Eppendorf Thermomixer and briefly centrifuged every 30 min of incubation time to minimize reaction condensation. Following the incubation, the reactions were stopped upon addition of SDS loading buffer, boiled and processed for Western blotting as described above.

Statistical Analysis. Statistical significance was determined using a two sample t-test assuming unequal variances. Any P value less than .05 was judged to be significant.

CHAPTER III: FATTY ACID OXIDATION IN THE FRATAXIN-DEFICIENT

HEART

Introduction

The mammalian heart primarily utilizes fatty acids as its preferred fuel

substrate, generating as much as 70% of the ATP for contraction from the β-

oxidation of fatty acids (*36*). Although the heart is capable of shifting its fuel

substrate during acute states of fasting, exercise, or β-adrenergic stimulation,

prolonged alterations in myocardial fuel utilization is associated with the evolution

of heart failure (*37*). Furthermore, individuals harboring genetic defects at any

step of the β-oxidation spiral are often intolerant to fasting and cold exposure and

are susceptible to sudden cardiac arrest (*211*). Genetic ablation of the NAD^+-

dependent deacetylase SIRT3 in mice causes accumulation of long-chain fatty

acid species in serum and tissue, intolerance to cold exposure, and a marked

inhibition of the β-oxidation enzyme LCAD (*66*), all symptoms which resemble

clinical disorders of β-oxidation. The finding that hyperacetylation in the Frataxin-

deficient heart is caused, at least in part, by SIRT3 dysfunction, suggests that

Frataxin deficiency may also reduce the capacity of the heart to oxidize fatty

acids. Additionally, mitochondrial disorders of the respiratory chain are known to

impair fat oxidation by redox state-mediated (low NAD/NADH) inhibition of

multiple NAD^+-dependent dehydrogenases of the oxidation spiral (*38*). Therefore,

the finding that SIRT3 may be impaired and that lack of Frataxin causes

deficiencies in multiple respiratory chain complexes both suggest that the

Frataxin-deficient heart experiences impaired oxidation of fatty acids, which could

have important implications for the clinical management of cardiomyopathy. We sought to test this hypothesis using the muscle creatine kinase Cre (MCK-Cre, MCK-KO) conditional mouse model of Friedreich's Ataxia.

Results

To test the hypothesis that fat metabolism is impaired in the Friedreich's Ataxia heart, staining of wild-type and MCK-KO ventricular myocardium was performed with the fat-soluble dye Oil-Red-O. The 10 week-old WT mice show no evidence of lipid in cardiomyocytes. In contrast, 10-week old MCK-KO mice exhibit an accumulation of ectopic lipid droplets throughout cardiomyocytes (Figure 3.1A). This cardiomyocyte steatosis was not observed in every cell, indicating some cardiomyocytes are more susceptible to this phenotype than others, which could reflect different areas of disease progression throughout the heart or mosaicism of the conditional Cre expressing allele. Cellular lipid primarily takes two forms; either triglyceride or free (non-esterified) fatty acids. Extracting total lipid and subsequently assaying for triglyceride content revealed no difference in the total cardiac triglyceride content between WT and MCK-KO animals (Figure 3.1C). This indicated that the ectopic lipid in MCK-KO hearts is probably composed of non-esterified fatty acid species and likely unoxidized acylcarnitine species consistent with mitochondrial dysfunction (*38, 212*). Analysis of protein expression in whole heart homogenate revealed no difference in the expression of the key fatty acid oxidation nuclear receptor PPARα or its
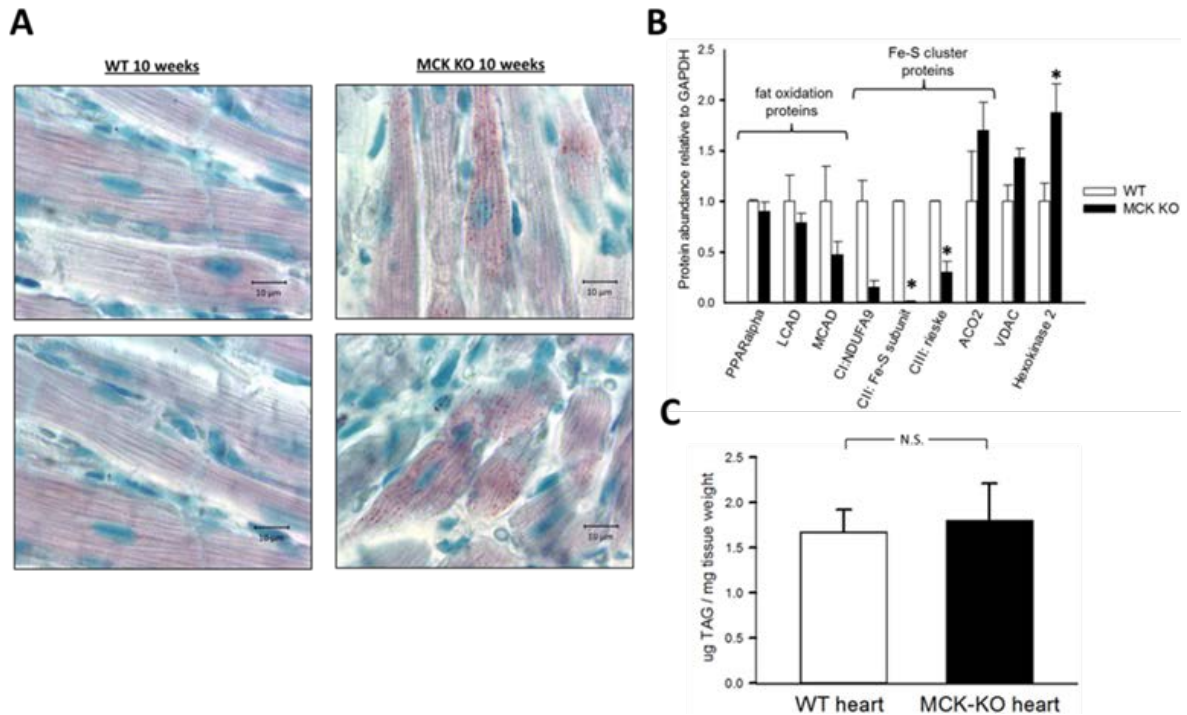
Figure 3.1 (A) Oil-Red-O staining of ventricular myocardium from wild-type and MCK-KO mice at 10 weeks of age. (B) β-oxidation and respiratory chain subunit protein expression in whole heart homogenate from WT and MCK-KO mice between 9 and 11 weeks of age (n=2-3/genotype, *$P<.05$) (C) Determination of cardiac triglyceride content in wild-type and MCK-KO mice between 9 and 10 weeks of age. N.S. = not significant.

downstream target genes LCAD or medium-chain acyl-CoA dehydrogenase (MCAD), but significantly decreased expression of respiratory chain complex II and III subunits SDHB and rieske protein, respectively (Figure 3.1B).

SIRT3$^{-/-}$ mice exhibit a fatty acid oxidation impairment caused by failure to deacetylate lysine 42 of LCAD in the β-oxidation spiral (66). Consequently, LCAD becomes hyperacetylated, which can be directly shown via mass-spectrometry or indirectly by immunoprecipitation of mitochondrial acetyl-lysine-containing proteins and subsequently Western blotting for LCAD. Performing the indirect

analysis on cardiac mitochondrial protein indicates that, similar to the *SIRT3*^{-/-}

mitochondria, Frataxin-deficient cardiac mitochondria show hyperacetylation of

the SIRT3 targets LCAD and AceCS2 (*121*) (Figure 3.2). Additionally, we found

another enzyme of β-oxidation, MCAD, to be hyperacetylated in the Frataxin^{-/-}

mitochondria. Together, these data suggested that altered protein acetylation

could underlie impairments in fat oxidation in the Frataxin-deficient-heart, but
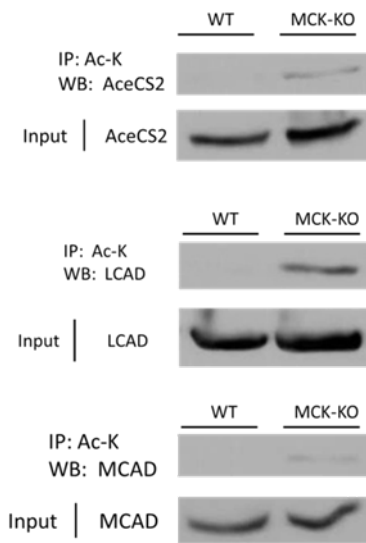
Figure 3.2 SIRT3 targets and enzymes of the fatty acid oxidation spiral are hyperacetylated the Frataxin-deficient heart. Mitochondrial acetyl-lysine containing protein were immunoprecipitated with an anti-acetyl-lysine containing antibody, resolved by SDS-PAGE and immunoblotted with antibodies raised against the mitochondrial proteins acetyl-CoA synthetase 2 (AceCS2), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain acyl-CoA dehydrogenase (MCAD).

does not exclude the possibility that LCAD and MCAD are generally

hyperacetylated at functionally irrelevant lysine residues.

To directly assay the capacity of Frataxin-deficient cardiac mitochondria to

oxidize fatty acids, we performed *ex vivo* analysis of fatty acid oxidation on

isolated cardiac mitochondria from WT and MCK-KO animals at 9-10 weeks of age using carbon-14 radiolabelled palmitate (*66*). Analysis of total ^{14}C- palmitate oxidation rate over a 60 min incubation period normalized to mitochondrial protein content revealed that total flux through the fatty acid oxidation pathway is decreased by 35% ($P< .05$, n=6-8/genotype) in Frataxin-deficient cardiac mitochondria relative to control mitochondria (Figure 3.3A). Extracting the ^{14}C-acid soluble metabolite fraction which represents incompletely oxidized downstream products of β-oxidation such as acetyl-CoA, citrate, organic acids, and short chain fatty acids, indicated, paradoxically, no difference between control and Frataxin-deficient cardiac mitochondria (Figure 3.3B). Further analysis of complete oxidation to carbon dioxide (CO_2) via capture of $^{14}CO_2$ with hyamine hydroxide indicated a highly significant 68% decrease ($P<.01$, n=4-5/genotype) in the rate of complete oxidation (Figure 3.3C). Taken together, these data show that Frataxin-deficient cardiac mitochondria suffer impaired β-oxidation of fatty acids, but, interestingly, the rate of complete oxidation to carbon dioxide is decreased to a greater extent than fat oxidation, resulting in a net accumulation of unoxidized acid-soluble metabolites as demonstrated by the unchanged ^{14}C signal in the acid-soluble metabolite fraction (Figure 3.4).

Discussion

These data demonstrate that Frataxin-deficient cardiac mitochondria suffer impaired oxidation of fatty acids despite no change in the expression of

key fat oxidation enzymes, which suggests the impairment may be

mechanistically related to protein post-translational regulation and/or metabolite
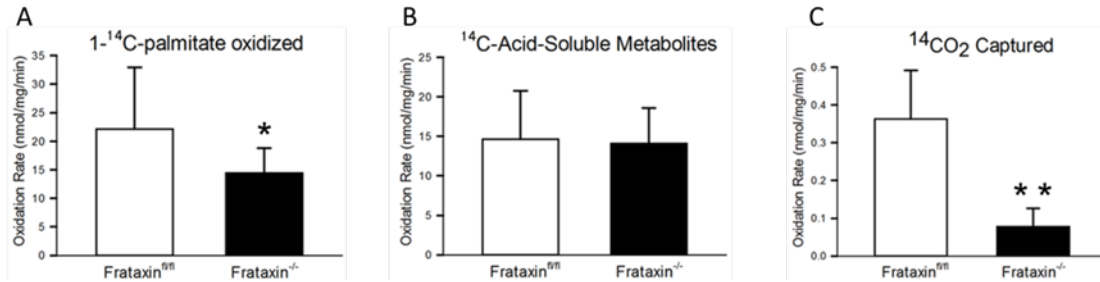
Figure 3.3 (A-C) Impaired fat oxidation, but accumulation of acid-soluble metabolites in Frataxin-deficient cardiac mitochondria. Isolated cardiac mitochondrial were incubated in the presence of carbon 14 radiolabelled palmitate and buffer supplemented with endogenous metabolites necessary for oxidation *in vitro* (see Materials & Methods) for 1 hour at 37°C. Following the incubation, perchloric acid was added which precipitates unoxidized fatty acid (A), maintains acid soluble metabolites (acetyl-CoA, organic acids) in solution (B), and liberates CO_2 which can be captured with hyamine hydroxide-saturated filter paper (C). All three parameters were measured via liquid scintillation counting. (n=4-8/genotype, *$P<.05$, **$P<.01$.)

feedback inhibition. Consistent with these explanations are data demonstrating

that *Fxn*^{-/-} mitochondria show hyperacetylation of the β-oxidation enzymes LCAD

and MCAD (Figure 4.2) and data presented in Chapter III demonstrating a

markedly decreased mitochondrial $NAD^+/NADH$ ratio. In order to convincingly

prove that altered fat oxidation is caused by protein hyperacetylation, it is

necessary to identify the functionally relevant acetyl-lysine residues on most, if

not all, the enzymes involved in fat oxidation and also generate site-specific

antibodies recognizing those relevant lysines and demonstrating that they are

either hyperacetylated or deacetylated; whichever condition is associated with

decreased activity of the enzyme. Thus, although protein hyperacetylation is associated with impaired fat oxidation in Frataxin-deficient mitochondria, it does not illustrate a causal relationship.
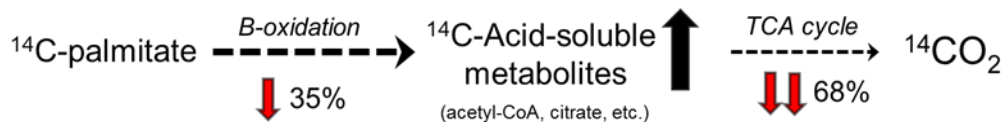
Figure 3.4 Schematic summarizing the experimentally determined fate of fatty acid-derived carbon in Frataxin-deficient cardiac mitochondria.

The finding that $Fxn^{-/-}$ mitochondria experience an accumulation of acid-soluble metabolites during substrate oxidation is very intriguing from the perspective of fundamental biochemistry. An accumulation of intermediary metabolites coupled with a dramatic reduction in the generation of CO_2 reflects a metabolic block in the first span of the TCA cycle. Consistent with these findings, cardiac Frataxin-deficiency is known to impair the activity of mitochondrial aconitase by 80-90% in human patients([18, 213]) and 70-74% in animal models ([15]). Frataxin directly regulates aconitase by converting its iron-sulfur cluster from the inactive $[3Fe-4S]^{1+}$ form to the active, $[4Fe-4S]^{2+}$ form in a citrate-dependent manner ([16]). Thus, assuming an oxidative mode of carbon utilization in cardiac mitochondria, the acid-soluble metabolites which have a high likelihood of accumulating are those directly upstream of aconitase in the TCA cycle, or citrate and acetyl-CoA. Future measurement of cardiac citrate and acetyl-CoA is warranted. Because acetyl-CoA is required for lysine acetylation, this data suggests than an accumulation of mitochondrial acetyl-CoA could be associated

with a dramatic increase in mitochondrial protein acetylation. Therefore, these findings led to the testing of this hypothesis in the following chapter.

Materials and Methods

Conditional knockout mouse breeding and genotyping. Wild-type and MCK-conditional knockout animals were bred and genotyped as described previously (*15, 59*). The primers used to detect the presence of exon 4 for genotyping were 5'-CTGTTTACCATGGCTGAGATCTC-3' and 5'-CCAAGGATATAACAGACACCATT-3' which generates a 500 bp product. The primers used to detect the presence of the Cre transgene were 5'-CCGGTCGATGCAACGAGTGAT-3' and 5'-CAAAGAGTCAT CCTTAGCGCC-3', which generate a 790 bp band. Mice that are genotyped as $Frda^{L3/\Delta}$, Cre+ represent the conditional knockout animals.

Mouse tissue preparation. For the isolation of whole heart homogenate, animals were sacrificed by CO_2 asphyxiation and the tissue was immediately excised, washed and minced in cold phosphate-buffered saline. The tissue was then placed in 1-2 ml of ice-cold complete RIPA buffer containing an EDTA-free protease inhibitor cocktail (Roche product #05056489001), 10 mM nicotinamide (NAM) and 200 nM Trichostatin A as deacetylase inhibitors. The tissues were then homogenized with at least 10 passes of a motor-driven Potter-Elvehjem homogenizer at high speed. The whole tissue homogenates were then transferred to 1.5 ml tubes and spun at 17,000 x *g* for 10 min at 4°C in a desktop centrifuge to pellet membranes and tissue debris. The supernatant was saved

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and immediately frozen on dry-ice and stored at -80°C for later protein quantification and Western blotting.

Tissue processing and Oil-Red-O (ORO) stain. Hearts were immediately excised and washed in cold PBS to remove blood. Hearts were fixed in 4% paraformaldehyde (PFA) with gentle shaking overnight at 4°C. Fixed tissue was removed from PFA and gently dabbed with a paper towel to absorb excess PFA. The tissue was then placed in a conical tube containing a 30% solution of sucrose in PBS at 4°C until the tissue fell to the bottom of the tube. Tissue was removed from the sucrose solution and gently dabbed with a paper towel to absorb excess sucrose. The tissue was then embedded in OCT (Tissue-Tek) medium, frozen in liquid nitrogen-cooled isopentane, and stored at -80°C until sectioning. After temperature equilibration in the cryostat, 10 micron tissue sections were cut onto positively charged slides. The tissue-containing slides were then placed in cold PBS for 3 min to remove excess OCT medium and then re-fixed in ice-cold 10% neutral-buffered formalin (NBF) for 3 min. The NBF was gently removed from the slide, which was then placed back in cold PBS for 1 min to rinse off excess NBF. The tissue was then placed in 100% propylene glycol (Sigma) for 3 min to avoid carryover of water into the ORO stain. The tissue was then stained in pre-warmed 0.5% ORO in propylene glycol for 30 min in a 60°C oven. Tissue was then differentiated in 85% propylene glycol for 2 min at room temperature followed by 2 rinses in distilled water. Slides were then quickly immersed in Gill's Hematoxylin (Polysciences, Gold Standard Series #3) for 2-3 seconds and then immediately placed in running tap water for 3-4 min. Following

the tap water rinse, the slides were placed back in distilled water before being mounted with coverslips and an aqueous mounting medium.

Cardiac triglyceride extraction and quantification. Tissue triglyceride extraction was performed using a modified Folch procedure substituting dichloromethane for chloroform (*214*). After evaporation of the organic phase, extracted triglycerides were resuspended in 100 µl of 1% Triton X-100 in isopropanol, briefly vortexed and incubated in an Eppendorf thermomixer for 5 min at 60°C and 1000 rpm to improve resolubilization of triglyceride. 2 µl of the re-solubilized sample was used for triglyceride quantification with the Infinity Triglyceride Reagent (Thermo) according to the manufacturer's instructions.

Immunoprecipitation of mitochondrial acetyl-lysine proteins. Reverse immunoprecipitation of acetyl-lysine containing mitochondrial proteins was performed as described previously (*185*).

Antibodies and Western Blotting. Antibodies used in this study include anti-acetyl-lysine (Immunechem), anti-acetyl-lysine (Cell Signaling),anti- PPARα (Novus Biologicals), anti-LCAD (Abnova), anti-MCAD, anti-aconitase, anti-complex II 30 kDa subunit, anti-NDUFA9, and anti-complex III Rieske protein (Mitosciences/Abcam), anti-SIRT3 D22A3, anti-VDAC D73D12, and anti-Hexokinase II (Cell Signaling), anti-Gapdh (Sigma). Following transfer of the SDS-PAGE gel proteins to nitrocellulose membranes, the membrane was blocked for 45 min in PBS-0.05% Tween-20 supplemented with 5% non-fat dry milk. Signals were visualized with SuperSignal West chemiluminescent substrate (Thermo-Pierce). Western blot signal intensities were determined by density

quantification using ImageJ software where appropriate. Protein concentrations were determined using the BCA method (Pierce).

***Ex vivo* measurement of fatty acid oxidation.** Flux through the fatty acid oxidation pathway in isolated cardiac mitochondria was measured following the method of Hirschey, et al. with one additional step to derive the fat oxidation rate from the amount of unoxidized 1-^{14}C palmitate (*66*) (also available at Protocol Exchange, http://www.nature.com/protocolexchange). After removing the acid-soluble supernatant from the reaction tube for scintillation counting, the remaining supernatant (approximately 200 µl) was discarded and residual acid was evaporated for 5 min in a speedvac. Following evaporation, unoxidized 1-^{14}C palmitate was extracted by incubating the acid-insoluble precipitate in 400 µl of 2:1 dichloromethane: methanol for 30 min at room temperature on a nutator. Then 200 µl of this extract was added to 4 ml of scintillation cocktail and counted. This extraction method consistently achieved greater than 97% recovery of unoxidized, acid-insoluble 1-^{14}C palmitate. The rate of oxidation was then derived by subtracting the counts of extracted 1-^{14}C palmitate from the total counts of reaction input and dividing by reaction incubation time.

CHAPTER IV: WIDESPREAD AND ENZYME-INDEPENDENT N^{ε}-ACETYLATION AND N^{ε}-SUCCINYLATION OF MITOCHONDRIAL PROTEINS VIA SPECIFIC BASE CATALYSIS

Abstract

Alterations in mitochondrial protein acetylation are implicated in the pathophysiology of diabetes, the metabolic syndrome, mitochondrial disorders, and cancer. However, the mechanisms initiating protein acetylation in mitochondria remain unknown. Here, by reproducing the physiologic pH and acetyl-CoA concentration of the mitochondrial matrix *in vitro*, we demonstrate dose- and time-dependent, but enzyme-independent N^{ε}-acetylation of mitochondrial proteins that is reversed by the SIRT3 deacetylase. In a similar manner, succinyl-CoA induces non-enzymatic N^{ε}-succinylation. These data suggest that protein acylation in mitochondria may be a form of chemical damage facilitated by the alkaline pH and high concentrations of reactive acyl-CoAs present in the mitochondrial matrix, and that SIRT3 evolved to mitigate the selectively inhibitory effects that non-enzymatic acetylation has on oxidative processes, especially during states of increased acetyl-CoA utilization such as fasting and caloric restriction. These findings may hold implications for the evolutionary roles that other sirtuin family members have in the maintenance of metabolic health and lifespan.

Introduction

In Chapter II it was demonstrated that the Frataxin$^{-/-}$ heart exhibits progressive hyperacetylation of mitochondrial proteins which is caused, at least in part, by a deficiency of the SIRT3 deacetylase. Directly comparing cardiac mitochondria from *SIRT3*$^{-/-}$ animals to Frataxin$^{-/-}$ animals via Western blot indicated that many hyperacetylated proteins are shared between the two conditions (Chapter II, Figure 2.7). However, in every instance of a shared band, the degree of hyperacetylation was greater in the *Fxn*$^{-/-}$ mitochondria. This observation lead supports the conclusion that complete absence of SIRT3 cannot account for the degree of acetylation observed in the *Fxn*$^{-/-}$ heart, and therefore there must be additional mechanisms, independent of SIRT3-dysfunction, that contribute to the hyperacetylation phenotype in these animals. This led us to initiate the following study which sought to test alternative mechanisms for mitochondrial protein hyperacetylation.

Understanding the molecular events underlying metabolic adaptations to fuel availability represents a fundamental challenge in cell biology with implications for the treatment of many diseases, such as cardiovascular disease, diabetes, and cancer. These cellular adaptations are often orchestrated through post-translational modification (PTM) of proteins, which can rapidly alter protein function to suit the metabolic circumstances. Recent proteomic investigations have revealed that one PTM, lysine acetylation (N$^{\varepsilon}$-acetylation), is widespread in bacterial and eukaryotic cells and likely has conserved adaptive and regulatory roles in diverse cellular processes (*64, 123, 124, 142, 215*). As the hub of cellular

metabolism and the primary site of acetyl-CoA utilization, mitochondria and the proteins within it are highly acetylated. The most recent survey of the mitochondrial protein acetylome indicates that over 65% of mitochondrial proteins are acetylated at over 2,100 sites (*216*). Focused studies show that N^ε-acetylation influences multiple processes of intermediary metabolism including fatty acid oxidation, the tricarboxylic acid cycle, oxidative phosphorylation and the urea cycle, among others (*66, 69, 217, 218*). Moreover, alterations in mitochondrial protein acetylation or an inability to dispose of excess mitochondrial acetyl-CoA are implicated in the pathophysiology of diabetes, the metabolic syndrome, and mitochondrial disorders such as Friedreich's Ataxia (*185, 219-221*). Despite the regulatory capacity and pervasive nature of acetylation in mitochondria, the mechanism(s) underlying acetyl-transfer in this organelle remain unknown.

Evidence from other cellular acetylation events suggests that non-enzymatic acetylation may occur in the conditions of the mitochondrial matrix. For example, members of the Gcn5-related N-acetyltransferase (GNAT) superfamily of enzymes employ a general base catalyst to deprotonate the positively charged ε-amino group of a lysine residue on the accepting protein (*222-224*). The deprotonated lysine then performs a nucleophilic attack on the carbonyl carbon in the acetyl group of acetyl-CoA, which displaces the thioester bond, leaving free –CoA and an N^ε-acetyl-lysine. Predictably, mutating the catalytic base of GCN5 abolishes acetyl-transfer, but more importantly, the rate of acetyl-transfer to histone substrate can be near completely recovered in the

absence of enzyme merely by increasing the pH of the reaction buffer (*224*). Thus, chemical (non-enzymatic) acetylation of lysine residues is energetically favourable provided acetyl-CoA is present and the pH is greater than 7.0 – a reaction which is well-documented (*224-228*) and is properly accounted for during kinetic characterization of acetyltransferases (*224*). Remarkably, the mitochondrial matrix maintains a higher acetyl-CoA concentration and higher pH than any other cellular compartment. Steady state oxidation of carbon fuel generates between 0.1 and 1.5 millimolar concentrations of acetyl-CoA within mitochondria (*229, 230*), which is 3 to 50-fold higher than that of the whole cell according to current estimates in yeast (*231*) (Figure 4.1B). Additionally, due to the extrusion of H^+ ions across the inner mitochondrial membrane to generate proton-motive force, the pH of the mitochondrial matrix is maintained at 7.9-8.0, which is 0.8 pH units higher than the next closest cellular compartment (cytosol, pH 7.2) (*232*). Although the ε-amino group of lysine has an intrinsic pKa of 10.5, it is well-established that local protein structure can promote marked decreases in amino acid pKa values via Coloumbic interactions with proximal charged residues (*233*) which, interestingly, are overrepresented among mitochondrial acetyl-lysine sequence motifs (*234*). Furthermore, non-enzymatic modification of lysine residues is known to occur *in vivo* (*235*) (Figure 4.1A). Taken together, these findings support the hypothesis that the chemical conditions of the mitochondrial matrix may uniquely facilitate non-enzymatic acetylation of proteins.

Results

   To test the hypothesis that mitochondrial N^{ε}-acetylation occurs chemically,

we incubated soluble liver mitochondrial protein with varying concentrations of

acetate or acetyl-CoA at pH 8.0. After incubating mitochondrial protein in 50 mM

acetate for 5 hours, there was no change in protein acetylation (Figure 4.2). In

contrast, incubating mitochondrial protein with low mM concentrations of acetyl-

CoA generated a dose- and time-dependent increase in lysine acetylation (Figure

4.1C-F). We reasoned that if a mitochondria-localized enzyme were responsible

for this acetylation, then heating the mitochondrial extracts to near-boiling prior to

incubating with acetyl-CoA would denature the enzyme thereby eliminating its

acetyltransferase activity. In contrast, if the observed increase in acetylation were

non-enzymatic, then protein denaturation would promote acetylation by

increasing the number of solvent-accessible lysines available to react with acetyl-

CoA. Strikingly, and consistent with the latter hypothesis, our data show that pre-

boiling the mitochondrial extracts resulted in a dose- and time-dependent

increase in lysine acetylation which was significantly greater relative to the native

(unheated) state (Figure 4.1C-F). Incubating native and denatured cardiac

mitochondrial protein with acetyl-CoA generated identical results (Figure 4.3).

This acetylation increase was not caused by the denaturation process, as

evinced by loss of acetylation while incubating denatured protein under acidic

conditions and was equally robust in a non-primary amine buffer system (Figure
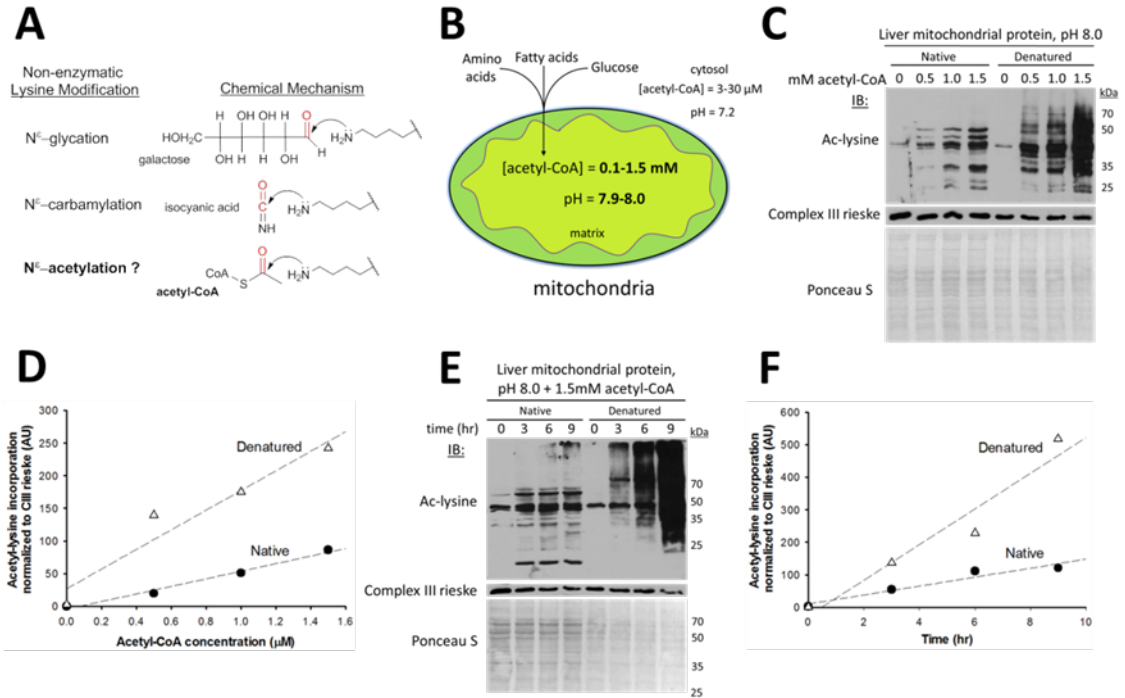
4.4).

Figure 4.1 Non-enzymatic N$^\epsilon$-acetylation in the chemical conditions of the mitochondrial matrix. (A) Non-enzymatic acetylation is chemically analogous to other endogenous non-enzymatic reactions like glycation and carbamylation. (B) Cartoon highlighting the unique chemical environment of the mitochondrial matrix. (C) Acetyl-CoA concentration-dependent increases in mitochondrial protein acetylation under native and denatured conditions. Mouse liver mitochondria were isolated, resuspended in buffer at pH 8.0 and 1.5 µg/µl, sonicated, and cleared by centrifugation. The extracts (30 µg) were incubated over a range of acetyl-CoA concentrations previously determined to occur in mitochondria (*229, 230*) (0.1-1.5 mM, 1 µl matrix volume/mg protein) for 6 hours at 37°C with gentle mixing. For denatured samples, mitochondrial extracts (30 µg) were heated for 10 min at 95°C prior to addition of acetyl-CoA. Samples were separated by SDS-PAGE and immunoblotted with a polyclonal acetyl-lysine antibody and a monoclonal Complex III rieske protein antibody, which served as a loading control. (D) Quantification of densitometry data in (c) normalized to Complex III. The control reaction with the smaller arbitrary value was set to zero and the remaining data points transformed accordingly. AU; arbitrary units. (E) Time-dependent incorporation of acetyl-groups into native mitochondrial protein lysine residues. (F) Quantification of the densitometry data in (e) normalized to Complex III. In (c) and (e), the nitrocellulose membrane used for immunoblotting was stained with the non-specific protein marker Ponceau S as indicated to show equal loading. IB, immunoblot; kDa, kilodaltons.
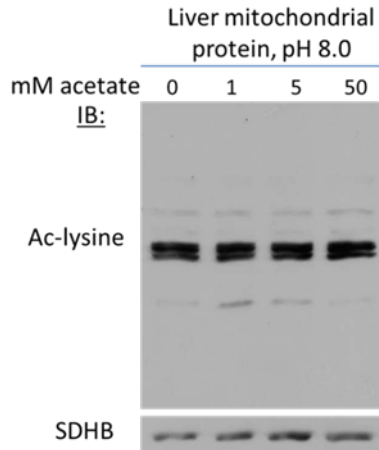
Figure 4.2 Free acetate does not influence the acetylation state of mitochondrial proteins. Incubating mitochondrial protein with millimolar concentrations of acetate has no effect on protein acetylation. Liver mitochondrial protein (30 μg) was prepared as described in materials and methods and incubated with the indicated concentrations of acetate at pH 8.0 for 5 hours at 37°C. Samples were resolved by SDS-PAGE and immunoblotted with a polyclonal acetyl-lysine antibody and a monoclonal succinate dehydrogenase subunit B antibody as a loading control. IB, immunoblot; SDHB, succinate dehydrogenase subunit B.

Although the increase in N^{ϵ}-acetylation in the denatured state showed that robust acetyl-transfer can occur in the absence of an enzyme in these conditions, it did not exclude the possibility of enzyme-mediated acetylation in the native state. Acetyltransferases in the GCN5 and MYST families are competitively inhibited by their product, coenzyme A (CoA) (*236*), and protein acetyltransferases outside the GCN5 and MYST families are predicted to undergo competitive inhibition owing to their structurally conserved CoA binding domains (*223*). Thus, if a protein acetyltransferase were responsible for acetyl-transfer in the native state, it would likely be competitively inhibited in the presence of CoA. To test the hypothesis that acetyl-transfer is competitively inhibited by CoA, we incubated native mitochondrial protein with increasing concentrations of CoA while keeping the acetyl-CoA concentration constant at

1.5 mM. As shown in Figure 4.5A, increasing the concentration of CoA to double

that of acetyl-CoA had no effect on the protein acetyl-transfer reaction. Thus,

acetyl-transfer under native protein conditions is not competitively inhibited by

CoA, and most likely occurs independently of CoA binding.
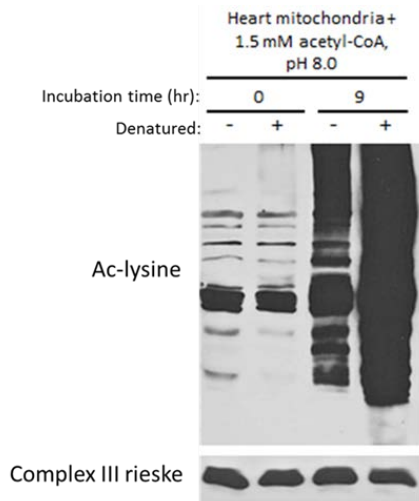
Figure 4.3 Acetyl-CoA induced acetylation of cardiac mitochondrial proteins under native and denatured conditions. Cardiac mitochondrial protein (30 µg) was prepared as described in materials and methods and incubated in 1.5 mM acetyl-CoA for 0 or 9 hours under native or denatured conditions as indicated. Samples were resolved by SDS-PAGE and immunoblotted with a polyclonal acetyl-lysine antibody and a monoclonal complex III rieske protein antibody as a loading control. IB, immunoblot

If protein acetylation in mitochondria is non-enzymatic, then incubating

non-mitochondrial protein under the minimal chemical conditions of the

mitochondrial matrix (pH 8.0, mM [acetyl-CoA]) should also induce protein

acetylation. Incubating non-acetylated bovine serum albumin (BSA) for 9 hours in

1.5 mM acetyl-CoA at pH 6 or 7 resulted in no, or minimal increases in protein

acetylation. In contrast, incubating BSA at pH 8.0 induced a striking increase in
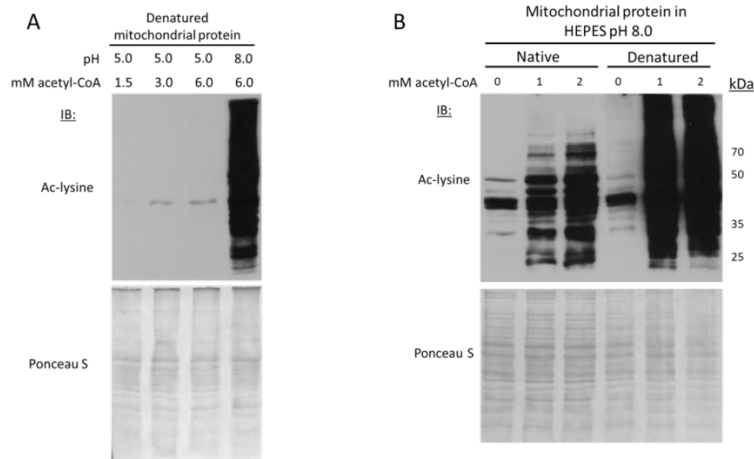
Figure 4.4 N$^{\varepsilon}$-acetylation of denatured mitochondrial proteins is not caused by the denaturation process and occurs equally well in a non-primary amine buffer. (A) Thirty micrograms of liver mitochondrial protein was denatured for 10 min at 95°C followed by incubation at 37°C with the indicated concentrations of acetyl-CoA at the indicated pH for 6 hours at 400 rpm on an Eppendorf Thermomixer. Samples were separated by SDS-PAGE followed by immunoblotting with a polyclonal acetyl-lysine antibody as described in materials and methods. Prior to the blocking step, the membrane containing transferred protein was imaged with the non-specific protein stain Ponceau S as seen below. IB, immunoblot. (B) Acetylation occurs equally well in a non-primary amine buffer. Liver mitochondrial protein were prepared as described in materials and methods, only in 50 mM HEPES (pH 8.0), 150 mM NaCl and incubated with the indicated concentrations of acetyl-CoA.

lysine acetylation (Figure 4.5B), which was further increased at higher pH and was directly proportional the concentration of hydroxide ions (Figure 4.6).

Recently, lysine succinylation was identified on mitochondrial and bacterial proteins, but a mechanism responsible for succinyl-transfer was not identified (*134, 237*). Using a validated succinyl-lysine antibody, we confirmed via Western blot that there are many succinylated proteins in mouse liver mitochondrial extracts (Figure 4.7A). Like acetyl-CoA, succinyl-CoA is an inherently reactive

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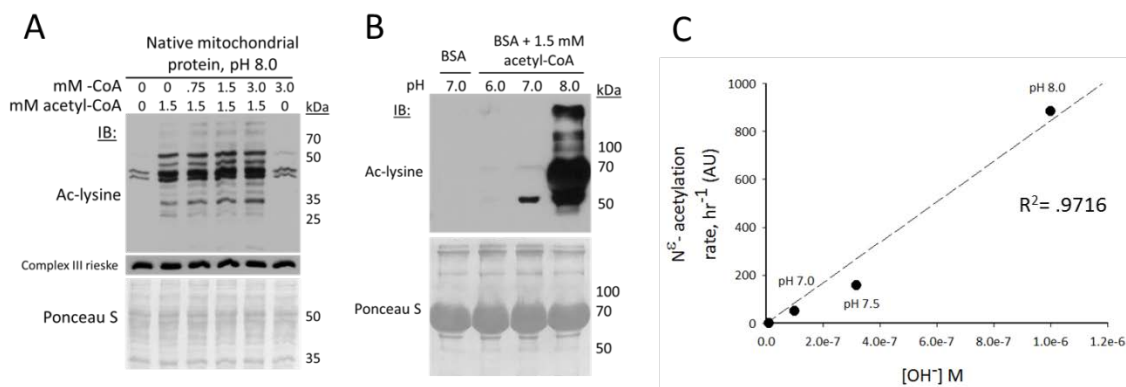
Figure 4.5 The acetyl-transfer mechanism is not competitively inhibited by CoA and its rate is directly proportional to hydroxide ion concentration. (A) Coenzyme A (CoA) does not competitively inhibit the observed acetyl-transfer reaction. Native mitochondrial protein was incubated with the indicated concentrations of CoA or acetyl-CoA at pH 8.0 for 6 hours and processed for immunoblotting as in Fig 1C. Complex III rieske protein was used as a loading control. (B) The chemical conditions of the mitochondrial matrix (pH 8.0, mM [acetyl-CoA]) are necessary and sufficient to induce widespread acetylation of a non-mitochondrial protein (bovine serum albumin, BSA) under native conditions. BSA was incubated in the presence of 1.5 mM acetyl-CoA at pH 6.0, 7.0, or 8.0 as indicated for 9 hours. Samples were resolved by SDS-PAGE and processed for immunoblotting as in Figure 1c. (C) Lysine acetylation rate is directly proportional to the concentration of hydroxide ions, consistent with specific base catalysis. AU; arbitrary units (derived from densitometry data in supplementary Figure 5.6).

short chain –CoA thioester that maintains steady-state concentrations in the

millimolar range (0.1-0.6 mM) in the mitochondrial matrix (Figure 4.7B) (*230*).

Accordingly, we hypothesized that incubating protein with succinyl-CoA at

physiological pH would initiate non-enzymatic succinylation. Consistently,

incubating non-acetylated BSA with 0.5 mM succinyl-CoA caused a pH-

dependent increase in lysine succinylation (Figure 4.7C). These results indicated

that the pH and acyl-CoA concentrations of the mitochondrial matrix are sufficient

to induce widespread and enzyme-independent protein lysine acylation. The

specific base-catalyzed nucleophilic acyl substitution mechanism predicted for

these reactions is summarized in Figure 4.8.
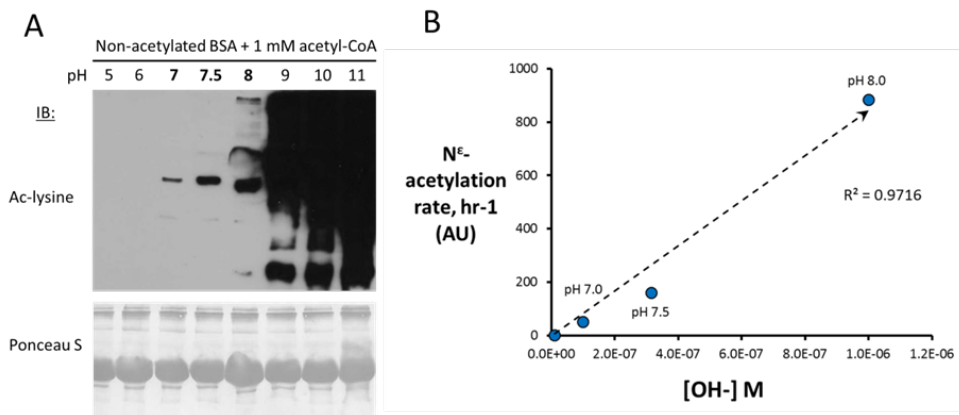
Figure 4.6 N^ϵ-acetylation of non-mitochondrial protein occurs at physiological pH and increases in a pH-dependent manner. (A) Twenty micrograms of non-acetylated bovine serum albumin (BSA) was incubated in 1 mM acetyl-CoA at the indicated pHs for 6 hours at 37°C as described in Materials and Methods. (B) Acetylation rate as a function of hydroxide concentration derived from densitometry of exposure above. The rate of N^ϵ-acetylation is directly proportional to [OH$^-$] prior to image saturation (< pH 9.0), consistent with specific base catalysis. Protein samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with a polyclonal acetyl-lysine antibody as described in Materials and Methods. IB, immunoblot.

The NAD$^+$-dependent deacetylase SIRT3 regulates global acetylation of

mitochondrial proteins (*234, 238*). Therefore, we hypothesized that SIRT3 target

proteins would become hyperacetylated in the presence of acetyl-CoA at pH 8

and that recombinant SIRT3 would mitigate the acetyl-CoA-induced increase in

mitochondrial protein acetylation. Immunoprecipitation and Western blotting of

acetylated mitochondrial proteins after incubation with acetyl-CoA showed that

the SIRT3 targets LCAD and NDUFA9 (*66, 238*) were hyperacetylated (Figure
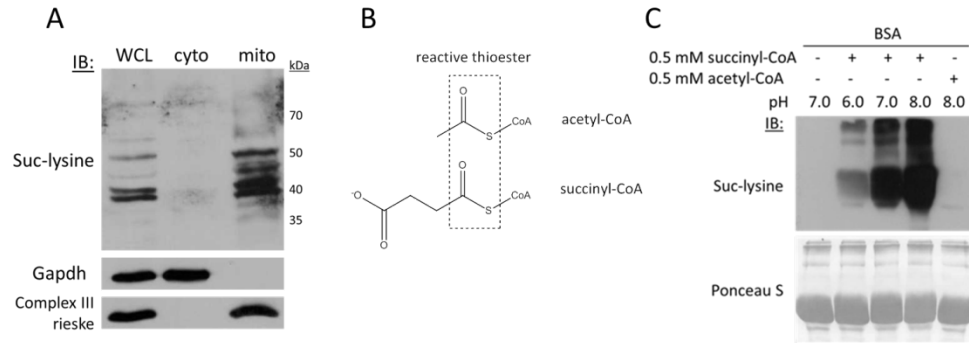
Figure 4.7 Protein succinylation is abundant within mitochondria and also occurs non-enzymatically at physiological pH. (A) Fractionation of mouse liver tissue into whole cell lysate (WCL), cytoplasm (cyto), and mitochondrial (mito) fractions followed by Western blotting with an anti-succinyl-lysine antibody. Gapdh and respiratory complex III rieske protein antibodies were used to confirm cytoplasmic and mitochondrial sub-fractions, respectively (B) Acetyl-CoA and succinyl-CoA are both inherently reactive –CoA thioesters. (C) The physiologic pH (8.0) and succinyl-CoA concentration (0.1-0.6 mM) (*230*) of the mitochondrial matrix are sufficient to induce lysine succinylation of protein under native conditions. Suc-lysine, succinylated-lysine.

4.9A). Furthermore, addition of recombinant SIRT3 in the presence of NAD$^+$ specifically reduced the acetyl-CoA induced increase in N$^\varepsilon$-acetylation; an effect which was lost upon addition of the sirtuin inhibitor, nicotinamide (NAM) (Figure 4.9B).

These data suggest that the tendency of a mitochondrial protein to become acetylated by acetyl-CoA will be dependent upon the dissociation constants, or pKa's, of the solvent-accessible lysine residues undergoing chemical acetylation. Although lysine residues have an intrinsic pKa of 10.5, residue pKas can be markedly altered via Coulombic interactions with proximally charged residues (*239*). Typically, a proximal negative charge will increase the pKa and a proximal positive charge will decrease the pKa of a lysine residue. A
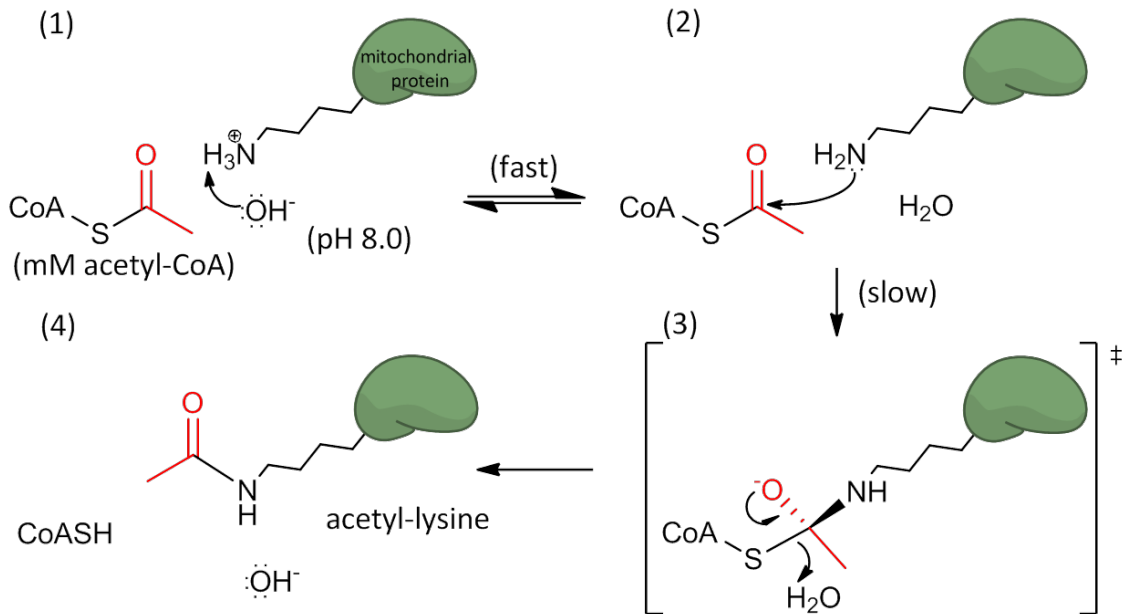
Figure 4.8 Specific base-catalyzed nucleophilic acyl substitution acetyl-transfer mechanism. Cartoon illustrating the specific base catalysed acetyl-transfer mechanism. The alkaline environment of the mitochondrial matrix reflects an increased concentration of hydroxide ions (OH⁻) free to abstract protons from solvent-accessible lysine residues (1). The deprotonated lysine can then perform a nucleophilic attack on the terminal carbonyl carbon of acetyl-CoA (2). A putative tetrahedral intermediate is formed (3), which then collapses, displacing the thioester bond, and leaving an acetylated lysine, CoASH, and hydroxide (4). This mechanism was adapted from that of the GCN5 acetyltransferase, which employs a general base catalyst to deprotonate lysine residues and initiate acetyl-transfer (*224*).

recent quantitative proteomics investigation catalogued all putative SIRT3 target

acetyl-lysine residues as well as the magnitude of acetyl-lysine change in

response to SIRT3 deletion (*234*). We hypothesized that putative SIRT3 target

residues undergoing the greatest increases in acetylation in response to SIRT3

deletion would have a greater number of proximal positively charged residues
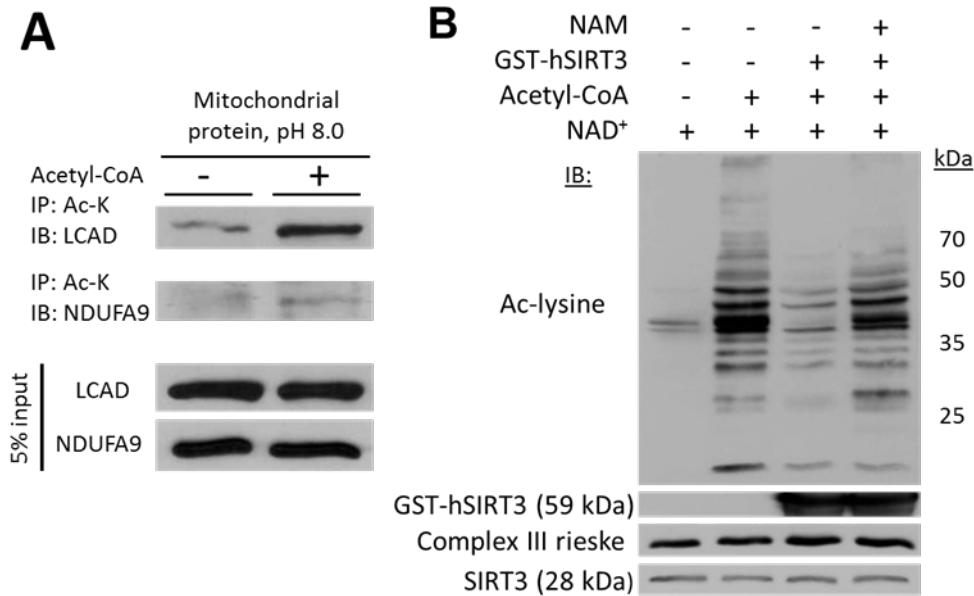
Figure 4.9 The SIRT3 deacetylase attenuates acetyl-CoA-induced acetylation of mitochondrial proteins. (A) Soluble liver mitochondrial extracts (120 µg) were incubated with or without 1.5 mM acetyl-CoA at pH 8.0 and 37°C for 4 hrs. Acetyl-lysine containing proteins were immunoprecipitated, followed by SDS-PAGE and immunoblotting for the SIRT3 targets NDUFA9 and LCAD. IP, immunoprecipitation; Ac-K; acetyl-lysine. (B) Liver mitochondrial extracts (30 µg) were incubated at pH 8.0 with acetyl-CoA, recombinant glutathione S-transferase (GST) tagged human SIRT3, and a sirtuin inhibitor, nicotinamide (NAM), in the presence of NAD^+ as indicated. Samples were resolved by SDS-PAGE and immunoblotted with a polyclonal acetyl-lysine antibody. A SIRT3 antibody was used to demonstrate the presence of recombinant (59 kDa) and endogenous (28 kDa) SIRT3; A respiratory Complex III rieske protein antibody was used as a loading control. IB, immunoblot; kDa, kilodaltons; NAD^+, nicotinamide adenine dinucleotide.

than target residues with smaller increases in acetylation. Using the proteomic data in the aforementioned study, we compared the number of positively charged residues within 2 amino acids of the target acetyl-lysine between 40 of the sites undergoing the greatest statistically significant increases in acetylation in

Low Positive Fold Change
2.00 ≤ Fold Δ ≤ 2.17

High Positive Fold Change
19.4 ≤ Fold Δ ≤ 99
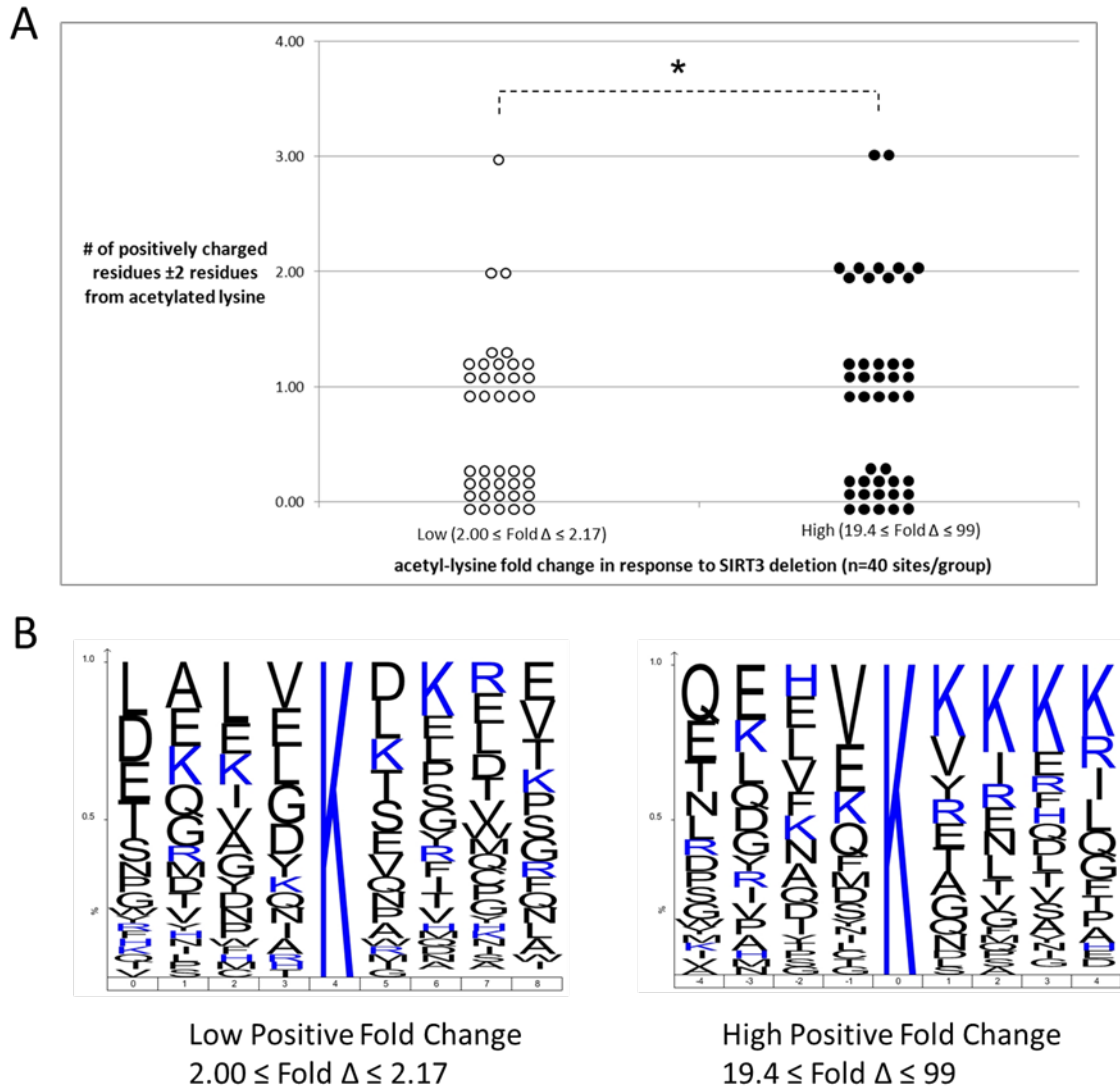
Figure 4.10 Mitochondrial protein acetylation sites undergoing the greatest acetylation increases in response to SIRT3 deletion have significantly more proximal positively charged residues, suggesting pKa perturbation. (A) Comparing the number of positively charged amino acids within 2 residues of the acetyl-lysine between acetyl-sites undergoing smaller statistically significant positive fold changes in response to SIRT3 deletion and those undergoing larger significant positive fold changes in response to SIRT3 deletion (*P<.05, Wilcoxon Rank Sums Test). (B) Relative occupancy of amino acids proximal to the acetylated site between the two groups. Positively charged residues are colored in blue and all others are black. The Y axis represents the total fraction of amino acid residues at the indicated position relative to the acetylated site.

95

response to SIRT3 deletion and 40 sites with lower statistically significant

increases in acetylation with a fold change greater than 2. As shown in Figure

4.10A, there were significantly ($P<.05$) more proximal positively charged amino

acids in the set undergoing the greatest increase in acetylation in response to

SIRT3 deletion, suggesting that local positively charged amino acids are lowering

the pKa of the SIRT3 target residues, thereby increasing the likelihood of non-

enzymatic acetylation at physiological pH. Analysis of proximal amino acid

abundance with ICE LOGO confirmed this finding (Figure 4.10B).

Discussion

    These data suggest that, in contrast to enzyme-mediated acetylation

present in the nucleus and cytosol where the pH and acetyl-CoA concentrations

are relatively low, acetylation of proteins in mitochondria may be a slow, non-

enzymatic event uniquely facilitated by an alkaline environment and millimolar

concentrations of acetyl-CoA. If this model holds true, then physiological and

pathological metabolic states which induce prolonged increases in mitochondrial

acetyl-CoA utilization would also promote hyperacetylation of mitochondrial

proteins. Consistent with this prediction are data showing that fasting, caloric

restriction, high fat diets, and chronic alcohol consumption all cause

hyperacetylation of hepatic mitochondrial proteins (*64, 123, 194, 220*). Moreover,

all are also associated with varying degrees of increased ketone body

production, which is stimulated, in part, by excess acetyl-CoA in hepatic

mitochondria (*240-243*). These findings strongly suggest that changes in the

mitochondrial concentration of acetyl-CoA during metabolic stress are a primary factor underlying altered protein acetylation in this organelle.

According to our proposed model, tissue-specific differences in mitochondrial protein acetylation will be dictated by several different physiological factors, the most influential of which are the number of solvent-accessible lysine residues within the mitochondrial proteome and the steady-state concentration of acetyl-CoA. Although every tissue contains mitochondria, each tissue has a different mitochondrial proteome with respect to both the types of proteins and their abundance. Therefore, each tissue-specific mitochondrion will have a different number of solvent-accessible lysine residues available to chemically react with acetyl-CoA. Differences in mitochondrial proteomes also influence the capacity of a mitochondrion to both generate and consume acetyl-CoA. Thus, distinct tissues will likely have different steady-state concentrations of mitochondrial acetyl-CoA which can fluctuate in response to metabolic stress such as fasting and, therefore, could also influence the non-enzymatic acetylation rate. Aside from solvent-accessible lysines and acetyl-CoA concentration, we predict that the rate of protein turnover, SIRT3 expression level, and degree of uncoupling will also influence tissue-specific differences in mitochondrial protein acetylation.

Recent studies indicate that the majority of acetylation sites identified in mitochondria may be non-functional. For example, among the eight acetylated lysines identified on the fatty acid oxidation enzyme long-chain acyl-CoA dehydrogenase (LCAD), only one acetylated site, lysine 42, altered enzyme

activity (*66*). Similarly, of the eleven acetylated lysines were identified on hydroxylmethylglutaryl CoA synthase 2, only 3 sites altered enzyme activity (*195*). Furthermore, this general trend was also observed for the mitochondrial enzymes acetyl-CoA synthetase 2, respiratory complexes I and II, manganese superoxide dismutase, isocitrate dehydrogenase 2, and malate dehydrogenase 2 (*69, 129, 206, 216, 217, 244*). Interestingly, the functionally relevant acetyl-lysines identified in these studies all decrease enzyme activity – an effect which is reversed via deacetylation mediated by the NAD^+-dependent SIRT3 deacetylase. Together, these studies suggest that only a fraction of mitochondrial acetyl-sites alter enzyme function and among those that do, the effect is consistently inhibitory.

In the liver, increased mitochondrial utilization of acetyl-CoA during caloric restriction correlates with an increase in mitochondrial protein acetylation and a parallel increase in SIRT3 deacetylase expression (*123, 216*). Similarly, both fasting and high-fat diet stimulate increased mitochondrial protein acetylation with a concurrent increase in SIRT3 expression (*64, 66, 220*). These findings indicate that metabolic states causing increased mitochondrial protein acetylation also stimulate the expression of the SIRT3 deacetylase. Despite the general increase in mitochondrial protein acetylation during caloric restriction, a number of protein lysine residues exhibit markedly decreased acetylation, and these same lysines show increased acetylation when SIRT3 is deleted, indicating these select residues are likely targets of SIRT3-mediated deacetylation (*216*). These studies, taken together with the present findings, suggest a model in which metabolic

states demanding increased acetyl-CoA utilization, such as fasting or caloric restriction, cause the mitochondrial concentration of acetyl-CoA to rise, which, in turn, favours non-enzymatic acetylation of proteins. Whereas increased acetylation has minimal consequences on protein function at the majority of solvent-accessible lysine residues, a subset of acetylated lysines located near active sites or relevant protein folds, compromise enzyme function and overall metabolic efficiency (*66, 195, 217*). Under these stressful conditions, mitochondrial NAD$^+$ levels rise (*139*) and SIRT3 deacetylase expression is induced (*66, 218*) in order to mitigate the selectively negative effects that acetylation confers on enzyme function and maintain metabolic efficiency. This proposed model is summarized in Figure 4.11.

Importantly, these studies do not preclude the existence of a mitochondrial acetyltransferase. Rather, they question what physiologic role a mitochondrial acetyltransferase would play considering the chemical conditions of the mitochondrial matrix are more than sufficient to initiate non-enzymatic acetylation. Our findings demonstrating that the chemical conditions of the mitochondrial matrix promote widespread non-enzymatic protein acetylation, and previous findings showing that the majority of acetyl-sites are functionally benign (*66, 69, 97, 195, 206, 216-218, 244*), supports the concept that protein lysine acetylation in mitochondria is, at least in part, a non-specific chemical consequence of acetyl-CoA metabolism, analogous to non-enzymatic N$^\varepsilon$-glycation of haemoglobin in diabetes (*235*). Furthermore, these findings may also have implications for another post-translational modification recently identified on
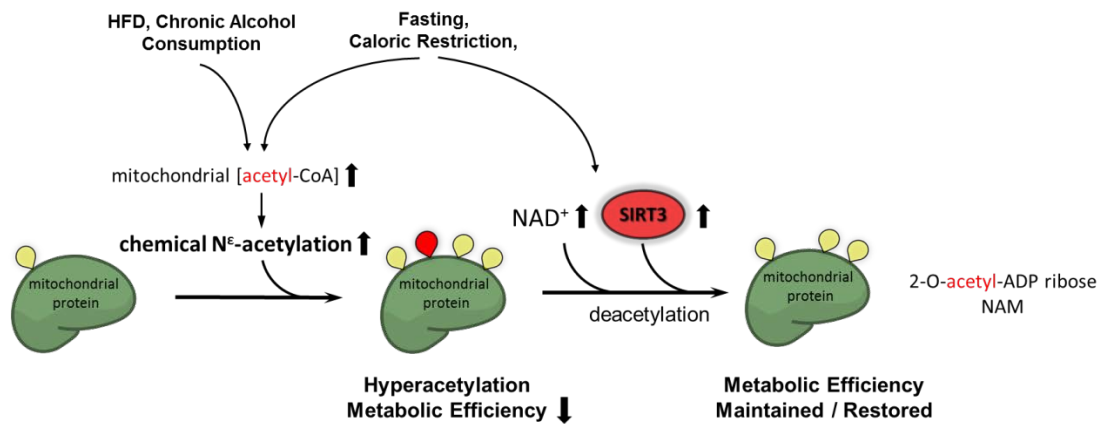
Fig 4.11 Proposed model for chemical acetylation and SIRT3-mediated deacetylation of hepatic mitochondrial proteins. Metabolic states demanding increased mitochondrial acetyl-CoA utilization (i.e., fasting, CR, HFD) raise the mitochondrial concentration of acetyl-CoA which promotes non-enzymatic acetylation of solvent-accessible lysine residues. While much of the acetylation has no apparent consequences for protein/enzyme function (yellow circles), a subset of acetylated lysines cause functional impairments and decrease metabolic efficiency (red circles) (*66, 97, 195, 206, 217*). Under these same conditions (fasting, CR, HFD) SIRT3 deacetylase expression is induced and NAD$^+$ levels rise (*139*) (fasting and CR only) to facilitate the deacetylation of functionally relevant acetyl-lysines and restore metabolic efficiency and homeostasis. The final reaction products are a deacetylated mitochondrial protein, 2-O-acetyl-adenosine diphosphate (ADP) ribose and nicotinamide (NAM). NAD$^+$, nicotinamide adenine dinucleotide; CR, caloric restriction.

mitochondrial proteins, lysine succinylation (*134, 245*). Like acetyl-CoA, succinyl-CoA is also present in millimolar concentrations in mitochondria (*229*), suggesting that lysine succinylation may also be a chemical event. Interestingly, although mitochondria-localized SIRT5 is a weak deacetylase, it has efficient lysine desuccinylase and demalonylase activity *in vitro (134).* These findings suggest that, in addition to their important roles as metabolic intermediates, excess acyl-CoA intermediates act as unintentional chemical donors – a

hypothesis first proposed in 1982 by Kirschenbaum (*246*). Thus, SIRT3 and SIRT5 may have evolved to harness the fasting-induced rise in mitochondrial NAD$^+$ (*139*) to minimize chemical acylation of protein caused by a concurrent increase in mitochondrial acyl-CoA utilization. Accordingly, and consistent with a recent review (*238*), we propose that the physiologic role of SIRT3 is as a quality control deacetylase which protects enzymes and proteins from chemical acetylation-induced impairment, especially during metabolic states that increase the mitochondrial pool of acetyl-CoA such as fasting, caloric restriction, or high-fat, ketogenic diets. More broadly, considering chemical acetylation of proteins is a slow, nonetheless, energetically favourable reaction at physiological pH (*224*) (Figures 4.5 and 4.6), it is tempting to speculate that an unexpected number of cellular acetylation events may be chemical in nature and certain sirtuins may have evolved to protect organisms from unintentional, yet unavoidable chemical acylation of cellular proteins.

Materials and Methods

Mitochondrial Isolation. Fresh tissue (100-200mg) from wild-type mice on an inbred mixed C57BL/6J x 129/Sv-ter background was dissected and immediately immersed in ice-cold mitochondrial isolation buffer (MIB)(220mM mannitol, 70 mM sucrose, 30 mM Tris-Cl (pH 7.4), 0.5 mM EGTA, and 0.1% BSA) supplemented with an EDTA-free protease inhibitor cocktail (Roche). Tissue was briefly minced with a razor blade and then homogenized in 1:5 w/v MIB with a 2ml hand dounce homogenizer (Liver=10 passes, Heart=30 passes).

Mitochondria were then isolated using a standard differential centrifugation method as described previously (*208*), then resuspended and frozen in 50 mM Tris-Cl (pH 8.0 at 37°C), 150 mM NaCl or 50 mM HEPES (pH 8.0), 150mM NaCl at 1.50 µg/µl. When ready for use, mitochondria were thawed on ice, sonicated twice for 15 seconds each on ice, then cleared by centrifugation at 12,000 x g for 10 min. Supernatants containing soluble mitochondrial protein were used for *in vitro* assays described below. Protein concentration was determined via the BCA method.

In vitro incubation of mitochondrial protein and BSA with acetyl-CoA, succinyl-CoA and Western Blotting. A 48 mM solution of acetyl-CoA (Sigma) and a 100mM solution of sodium acetate were each made in 50 mM Tris-Cl, 150 mM NaCl and adjusted to pH 8.0 at 37°C. Serial dilutions were made using 50 mM Tris-Cl (pH 8.0 at 37°C), 150 mM NaCl as the diluent. Twenty microliters of the appropriate dilution was added to 20 µl of soluble mitochondrial protein (1.5 µg/µl) in 50 mM Tris-Cl (pH 8.0 at 37°C), 150 mM NaCl to achieve the desired concentration of acetyl-CoA, or acetate. Total reaction volumes of 40 µl were incubated for 6 hours at 37°C at 400 rpm in an Eppendorf Thermomixer unless otherwise indicated. Reaction tubes were briefly centrifuged during the incubation to minimize condensation. For denatured protein experiments, soluble liver mitochondrial protein was heated at 95°C for 10 min and cooled on ice prior to the addition of acetyl-CoA. To test for competitive inhibition by CoA, a 12 mM solution of CoA (Sigma) was made in 50 mM Tris-Cl, 150 mM NaCl, and adjusted to pH 8.0 at 37°C. Dilutions of this CoA solution and acetyl-CoA solutions were

added to 30 μg of native mitochondrial protein in 50 mM Tris-Cl (pH 8.0 at 37°C), 150 mM NaCl to achieve the desired concentrations in a final volume of 40 μl and then incubated for 6 hours at 37°C and 400rpm in an Eppendorf Thermomixer. For BSA experiments, 2 μg/μl solutions of non-acetylated, fatty-acid free BSA were made in 50 mM Tris-Cl, 150 mM NaCl and adjusted to either pH 6.0, 7.0, or 8.0 at 37°C. Twenty microliters of each BSA solution was added to 20 μl of a 3 mM solution of acetyl-CoA or 1 mM Succinyl-CoA adjusted to the same pH for final conditions of 40 μl total volume, 1.5 mM acetyl-CoA or 0.5 mM succinyl-CoA, and 1μg/μl BSA in 50 mM Tris-Cl, 150mM NaCl at pH 6, 7, or 8. BSA reactions were incubated for 9 hours as described earlier, or 6 hours for succinyl-CoA. Following the incubations, loading buffer was added and the samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, blocked, and probed with a polyclonal acetyl-lysine antibody (Cell Signaling) or a polyclonal succinyl-lysine antibody (PTM Biolabs, Inc.), and monoclonal antibodies against succinate dehydrogenase iron-sulfur subunit (SDHB) and complex III rieske protein (UQCRFS1)(Mitosciences/Abcam). Signal was visualized with HRP-conjugated polyclonal goat-anti-rabbit or goat-anti-mouse secondary antibodies and Supersignal Pico West chemiluminescent substrate (Pierce) and exposed to X-ray film. The pH was adjusted with an Oakton 1100 series digital pH meter or Corning pH meter 240 electrode calibrated to the same set of Fisher Scientific buffer solution standards.

Immunoprecipitation. Mitochondrial protein extracts were prepared as described above and 120 μg per sample was incubated with or without 1.5 mM

acetyl-CoA at pH 8.0 and 37°C for 4 hrs. After incubation, the samples were diluted 1:10 in 50mM HEPES (pH 7.1), 150mM NaCl and 25 µl of acetyl-lysine antibody conjugated agarose beads (Immunechem) were added to each sample followed by incubation for 18 hrs at 4°C with gentle rocking. Beads were centrifuged at 400 x g for 1 min at 4°C and the supernatant decanted. The beads were washed 3 x 5 min in 250 µl of 50 mM Tris-Cl (pH 7.25), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40 and then boiled in loading buffer prior to SDS-PAGE.

In vitro SIRT3 deacetylation assay. Soluble liver mitochondrial protein (30µg) was incubated in the following final reactions conditions: 48.4 mM Tris-Cl (pH 8.0 at 30°C), 162 mM NaCl, 5.7% glycerol, 1.1 mM $MgCl_2$, 5.7 mM NAD^+, ± 7.2 mM Acetyl-CoA, ± 3µg GST-hSIRT3, ± 10 mM nicotinamide in a total volume of 70 µl. Reactions were incubated for 4 hrs at 30°C in an Eppendorf Thermomixer set to 400 rpm. Reactions were briefly centrifuged at 2 hrs to minimize reaction condensation. Reactions were stopped with addition of loading buffer, and resolved on a 12% SDS-PAGE followed by immunoblotting with acetyl-lysine and complex III rieske protein antibodies as described earlier, and a polyclonal SIRT3 antibody (Cell Signaling).

Image Analysis. Integrated densitometries of Western blot exposures were calculated using Image J and a simple linear regression was performed on the plotted values (Figure 5.6). Cartoons and figures were generated using Chembiodraw Ultra 12.0 and Microsoft PowerPoint.

CHAPTER V: CONCLUSIONS

Friedreich's Ataxia (FRDA) is a fatal progressive mitochondrial disease caused by a pathological deficiency of the mitochondrial iron-binding protein Frataxin. Although the cause of FRDA is known, the disease has no cure, reliable treatment, or useful biomarker. Although the typical presenting symptom is ataxia, the most common cause of death among FRDA patients is heart failure, which represents a poorly understood, understudied, and poorly managed aspect of this devastating disease (*247*). Due to the general lack of clinical and basic knowledge regarding the cardiomyopathy in FRDA, many important questions remain.

The majority of the pathological mechanisms operating in FRDA originate from mitochondrial dysfunction. However, few studies have focused on the mitochondrial and metabolic consequences of cardiac Frataxin deficiency. Recently, acetylation of mitochondrial proteins has been implicated in global nutrient sensing and the coordination of metabolic flux, which suggests altered protein acetylation could mediate pathological changes in mitochondrial metabolism (*39*). Understanding these potential metabolic changes could provide novel insight into the mechanisms underlying FRDA cardiomyopathy as well as potential metabolic biomarkers which could be used to study disease progression and/or response to treatments. This study has investigated the role of the post-translational protein modification lysine acetylation in the evolution of cardiomyopathy in two conditional mouse models of FRDA. The data demonstrates a late-onset, progressive mitochondrial protein hyperacetylation in

the Frataxin-deficient heart. This novel disease phenotype develops concurrently

with cardiac hypertrophy, is associated with a marked shift in mitochondrial redox

state (NAD$^+$/NADH) and parallels the previously reported progressive decline in

mitochondrial iron-sulfur cluster dependent enzyme activity (*15*).

The NAD$^+$-dependent deacetylase SIRT3 regulates global acetylation of

mitochondrial proteins and is particularly important in the maintenance of

oxidative and intermediary metabolism (*69, 125, 234*). Mitochondrial protein

hyperacetylation and loss of SIRT3 promotes the development of multiple

pathologies including diabetes, the metabolic syndrome, heart failure, hearing

loss, and cancer (*127, 182, 203, 219, 220*). These studies suggest that SIRT3-

deficiency and mitochondrial protein hyperacetylation are pathological in nature.

Upon investigating the hypothesis that the dominant mitochondrial NAD$^+$-

dependent deacetylase SIRT3 is deficient in FRDA, we found that SIRT3 targets

are hyperacetylated, SIRT3 is excessively carbonylated, and recombinant SIRT3

can reverse the protein hyperacetylation phenotype *in vitro*, all suggesting a

defect in endogenous SIRT3 activity which could contribute to cardiomyopathy in

FRDA. However, directly comparing SIRT3 knockout cardiac mitochondria to

Frataxin KO cardiac mitochondria indicated that there must be mechanisms

independent of SIRT3 deficiency contributing to the hyperacetylation phenotype

in the Frataxin-deficient heart. Furthermore, *ex vivo* analysis of fatty acid

oxidation in cardiac mitochondria revealed a defect in fat oxidation which should

be confirmed through Positron Emission Tomography of FRDA patient hearts *in*

vivo using radiolabelled substrate tracers. This analysis also revealed an

accumulation of intermediary metabolites, suggesting that mitochondrial acetyl-CoA abundance could be mediating the hyperacetylation phenotype. This triggered the investigation of the basic mechanism underlying protein lysine acetylation in mitochondria, which is unknown.

Protein acetylation in mitochondria is a relatively new field of investigation and many important basic questions remain. It is well-established that enzyme-mediated lysine acetylation events have important roles in gene regulation and signaling events in cellular organisms. However, it is also known that the intracellular metabolite required for lysine acetylation, acetyl-CoA, is inherently reactive and capable of initiating non-enzymatic lysine acetylation events at physiological pH (*224, 225, 227, 248*). Surprisingly, despite the ubiquitous nature of acetyl-CoA and protein acetylation in living organisms, virtually no work has investigated the possibility that non-enzymatic lysine acetylation occurs and has functional consequences in biological systems.

In vitro studies have shown that non-enzymatic acetylation is favored at alkaline pH and is acetyl-CoA concentration-dependent (*224, 227*). Thus, it was logical to hypothesize the mitochondrial matrix, which maintains a more alkaline pH and higher acetyl-CoA concentration than any other cellular compartment, might be one cellular environment in which non-enzymatic protein acetylation would be widespread (*232*). The present data strongly suggest that non-enzymatic protein acetylation occurs in mitochondria and that the biological purpose of the NAD^+-dependent deacetylase SIRT3 is to minimize its deleterious functional consequences. Furthermore, because SIRT5 is a mitochondria-

localized desuccinylase, succinyl-CoA achieves high concentrations within mitochondria, and protein succinylation is abundant within mitochondria (Figure 4.7), is logical to suggest that SIRT5 may have evolved to cope with the consequences of non-enzymatic succinylation. The common theme suggested by these findings is that many short chain acyl–CoA thioesters present within cells have the potential to non-enzymatically modify proteins, and thereby alter their function. This hypothesis was presciently proposed in 1982, but technology and reagents at the time were insufficient to properly investigate its possibility (*246*). Interestingly, mitochondria-localized SIRT4 currently lacks a defined enzymatic activity, but it is tempting to speculate that it serves to remove another short-chain acyl moiety from lysine residues, which likely occurred non-enzymatically. These studies have provided a viable mechanism for protein acetylation in mitochondria which is related to the distinct chemical environment of the mitochondrial matrix, however these results do not exclude the possibility of an enzyme-mediated acetylation mechanism in mitochondria. It is, however, an indisputable empirical fact governed by the deterministic laws of chemistry that non-enzymatic acetylation of protein lysine residues will occur at physiological pH, and especially at mitochondrial pH, in the presence of acetyl-CoA.

Biology is inherently messy. There are numerous examples including error-prone DNA polymerases and accidental phosphorylation during glycogen synthesis. However, it is this messiness that is the driving force of evolutionary innovation (*249*). The data contained herein suggest that the NAD^+-dependent

deacetylases SIRT3, SIRT5, and perhaps other Sirtuins, evolved to mitigate the deleterious metabolic consequences associated with non-enzymatic protein acylation caused by the inherent reactivity of -CoA thioesters, and thus may be considered damage control enzymes.

A key implication of these findings is that acetyl-CoA, the most fundamental two-carbon unit of amino acid, fatty acid, and carbohydrate catabolism, may be, at least within mitochondria, non-enzymatically proteotoxic. Thus, perhaps, it is not surprising that genetic ablation of the major mitochondrial deacetylase SIRT3 is associated with multiple age-related pathologies including the metabolic syndrome, diabetes, cancer, and a general impairment in mitochondrial and metabolic homeostasis. This interpretation, although controversial, could perhaps, be integral to understanding why the Sirtuin family of deacetylases are believed to mediate the beneficial and healthful effects of caloric restriction and why they are protective in various metabolic diseases (*71, 114*).

How are these results relevant to the mitochondrial protein hyperacetylation phenotype and pathogenesis of cardiomyopathy in FRDA and perhaps other mitochondrial cardiomyopathies? Our results suggest that Frataxin-deficient cardiac mitochondria experience coordinate post-translational suppression of oxidative metabolism which is, in itself, pathological, because the heart is heavily dependent on oxidative processes for the ATP production necessary for cardiac contraction. However, the cause of this cannot be fully explained by SIRT3 impairment. This suggests a pathological process related to

the mechanism of acetylation in mitochondria, such as excessive acetyl-CoA

within mitochondria, and/or additional impairments in, as yet, unidentified

mechanisms of deacetylation. Previously, Vyas *et al.* reported that exogenous

protein replacement therapy with Frataxin fused to the HIV transactivator of

transcription (TAT-Frataxin) can partially rescue and extend lifespan in the NSE-

Cre Frataxin conditional knockout animals (*59*). Analyzing protein acetylation in

preliminary data from NSE-Cre animals treated with TAT-Frataxin suggests an

attenuation of protein acetylation in the Frataxin-deficient animals treated with

TAT-Frataxin. This suggests that Frataxin protein replacement therapy can

oppose the mitochondrial protein acetylation process, likely through indirect
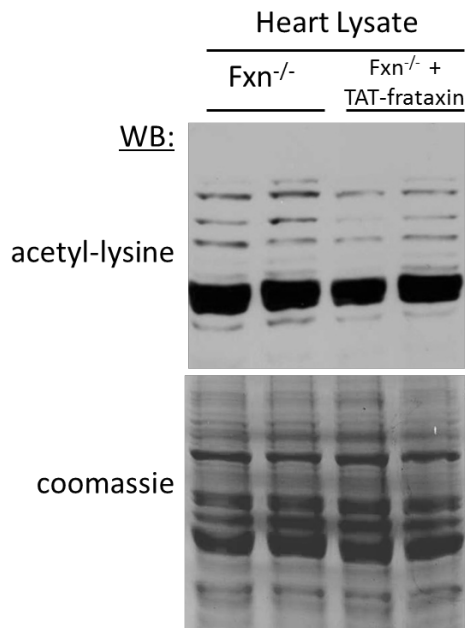
Figure 5.1 The effect of TAT-Frataxin protein replacement therapy on cardiac protein acetylation status. Western blot probing for acetyl-lysine residues on whole heart lysate (40 µg) derived from 25-30 day old NSE-Cre Frataxin conditional knockout animals or NSE-Cre animals treated with TAT-Frataxin for 3 weeks.

mechanisms, and further supports TAT-Frataxin as a viable treatment option for FRDA.

Future research should address if the mitochondrial protein hyperacetylation can be reduced through transgenic overexpression of SIRT3 and if this intervention can improve cardiac function or extend lifespan in the conditional mouse models of FRDA. It is also possible that this intervention will have no salutary effects, because it will not serve to correct multiple iron-sulfur cluster defects. Future research should also focus on rigorous metabolomic characterization of both the Frataxin-deficient heart using animal models and serum samples from FRDA patients as well as cardiac positron emission tomography imaging of FRDA patients using substrate tracers. Together, this work will offer comprehensive understanding of the various mitochondrial and metabolic abnormalities in FRDA, which will further enhance our understanding of disease mechanisms and provide candidate biomarkers for monitoring disease progression and treatment response.

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249. D. S. Tawfik, Messy biology and the origins of evolutionary innovations. *Nat. Chem. Biol.* **6**, 692 (Oct, 2010).

CURRICULUM VITAE

Gregory Randall Wagner

Education

Ph.D. (2008-2013)
Indiana University-IUPUI, Indianapolis, IN, Major: Medical and Molecular
Genetics, Minor: Life Sciences
GPA 3.90/4.0

Bachelor of Arts (2004-2008)
Depauw University, Greencastle, IN, Major: Biochemistry
GPA 3.54/4.0

Honors, Awards, Fellowships

-American Heart Association Pre-doctoral Fellowship, 2011-2013
-Full international travel scholarship, 2011 Friedreich's Ataxia Research Alliance
scientific conference held in Strasbourg, France
-IU School of Medicine travel grant to present at Keystone Symposia, 2012
-Invited Lecture, IUSM Diabetes Research Group, 2012
-Merit-based Travel Grant, IU School of Medicine, 2008-2009
-Science Research Fellow, Depauw University, 2004-2008

Professional Experience

Graduate Student, Indiana University School of Medicine, Dept. of Medical &
Molecular Genetics, Indianapolis, IN, August 2008-present
        -Developed an independent research project and wrote a grant that
        resulted in external funding through the American Heart Association
        -Research in molecular biology and biochemistry, working knowledge of
        metabolism, bioenergetics, and cardiovascular biology
        -Published two 1st author articles, one 2nd author article, and one middle
        author article

Summer Research Intern, Depauw University, Greencastle, IN, June 2006-
August 2006
        -Inorganic chemistry, synthesized novel Copper-Cerium polymer
        complexes in ionic liquids, mentor: Dr. Hilary Eppley

Summer Research Intern, Depauw University, Greencastle, IN, June 2005-
August 2005
        -Biology/Biochemistry, cloned, sequenced, and characterized serotonin
        receptors in Zebrafish, mentor: Dr. Henning Schneider

Conferences Attended

1. 2013 Keystone Symposia: Mitochondria, Metabolism, and Myocardial Function. Keystone, CO
2. 2012 American Heart Association Scientific Sessions. Los Angeles, CA
3. 2011 Childhood Ataxia Conference. Savannah, GA
4. 2011 Friedreich's Ataxia Research Alliance Conference. Strasbourg, France
5. 2010 Midwest Pediatric Cardiology Conference. Indianapolis, IN
6. 2010 American Heart Association Scientific Sessions. Chicago, IL
7. 2006 American Chemical Society Meeting. Atlanta, GA

Publications

1. **Wagner GR**, Payne RM "Widespread and enzyme-independent N^ε-acetylation and N^ε-succinylation of protein in the chemical conditions of the mitochondrial matrix ," *under revision.*

2. **Wagner GR**, Pride PM, Babbey CM, Payne RM "Friedreich's Ataxia reveals a mechanism for coordinate regulation of oxidative metabolism via feedback inhibition of the SIRT3 deacetylase," *Human Molecular Genetics*, 2012 Jun 15; 21(12):2688-97. Epub 2012 Mar 6. PubMed ID: 22394676

3. **Wagner GR**, Payne RM "Mitochondrial Acetylation and Diseases of Ageing," *Journal of Ageing Research,* vol. 2011, Article ID 234875, 13 pages, 2011. PubMed ID: 21437190

4. Payne RM, **Wagner GR** "Cardiomyopathy in Friedreich's Ataxia: clinical findings and research," *Journal of Child Neurology*, 2012 Sep; 27(9):1179-86. Epub 2012 Jul 4. PMID: 22764179

5. Zhang W, Chen H, Wang Y, Yong W, Zhu W, Liu Y, **Wagner GR**, Payne RM, Field LJ, Xin H, Cai CL, Shou W. "Tbx20 transcription factor is a downstream mediator for bone morphogenetic protein-10 in regulating cardiac ventricular wall development and function," *Journal of Biological Chemistry* 2011 Oct 21; 286(42):36820-9. PubMed ID: 21890625

Abstracts

1. **Wagner GR**, Pride PM, Payne RM "A mitochondrial cardiomyopathy reveals a mechanism for coordinate regulation of oxidative metabolism" 2013 Keystone Symposia: Mitochondria, Metabolism, and Myocardial Function. Keystone, CO

2. **Wagner GR** and Payne RM. Cardiac Mitochondrial Proteins are Hyperacetylated in a Mouse Model of Friedreich's Ataxia. *Circulation*. 2010; 122:A19675. Abstract No. 19675, Session: Genetically Engineered Models of Human Disease. 2010 American Heart Association Scientific Sessions, Chicago, IL

3. Zhu W, Reuter S, Zhang W; **Wagner GR**, Becker A, Payne RM, Wei L, Caldwell R, Shou W, Field LJ. The Pivotal Role of p53 in Doxorubicin-Induced Acute versus Chronic Cardiotoxicity. *Circulation.* 2011: 124:A16127. Abstract No. 16127, Session: Regulation of Cell Death Signaling Pathways. 2011 American Heart Association Scientific Sessions, Orlando, FL

Activities

Consulting experience for Indiana University Research and Technology Corporation, Fall 2012

Designed and Initiated "Research Protocols Summer Seminar" series through Genetics Dept., 2011

IBMG Ph.D. program recruitment weekend volunteer, 2010-2012

IBMG Ph.D. program student mentor, 2009-2010

PNAS peer reviewer

IU School of Medicine student travel awards peer reviewer, 2011

Cardiac Group Student-Post-Doc PPG Seminar Series Selection Committee, 2009