NOVEL ROLES FOR IRON SULFUR CLUSTERS IN RED BLOOD CELL DEVELOPMENT AND HUMAN DISEASE

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ABSTRACT

Iron-sulfur (Fe-S) clusters are versatile enzyme cofactors that are essential to all living organisms due to their diverse electron transfer and catalytic capabilities. In this collection of studies, we investigated the function of Fe-S clusters in several physiological settings that are relevant to human health and disease. We identified cellular conditions in which the biosynthesis of heme, the iron-containing cofactor present in hemoglobin and other enzymes, may be directly influenced by cellular Fe-S cluster assembly processes. This interdependence of the heme biosynthesis and Fe-S cluster assembly pathways relied on the provision of Fe-S clusters to the terminal heme biosynthetic enzyme, ferrochelatase. We also studied the tissue specificity and pathophysiology of a rare inherited human disease known as ISCU Myopathy, caused by deficiency of Fe-S clusters due to low expression of the Fe-S scaffold protein, ISCU. We found that tissue-specific splicing and oxidative stress lead to pathological depletion of ISCU protein in patient skeletal muscle, while other patient tissues are spared, perhaps in part because they express higher levels of ISCU protein. We performed global gene expression analysis on ISCU Myopathy patient skeletal muscle biopsies, and found evidence for a coordinated pattern of changes in biochemical pathways and muscle tissue architecture due to chronic Fe-S cluster deficiency. These changes included an increase in the proportion of oxidative muscle fibers.
relative to glycolytic fibers and enhanced capillary perfusion, providing a possible basis for the muscle hypertrophy observed in these patients. We also found evidence for activation of a starvation response and ketogenesis in patient muscles, which probably reflected a coordinated response to cellular energy starvation and over-accumulation of acetyl-coenzyme A. Finally, we found that ISCU Myopathy patient muscles synthesize and secrete elevated levels of the starvation response hormone FGF-21, leading to measurable increases in FGF-21 levels in patient blood samples. Our findings suggest that FGF-21 may serve an important future role in diagnosing mitochondrial myopathies, and in assessing potential therapeutic treatments. Together these studies illuminate the role occupied by Fe-S clusters in the interface between inorganic chemistry and human health, and demonstrate novel roles and future possibilities for these ancient cofactors.
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First and foremost I would like to thank my mentor, Dr. Tracey Rouault, for providing me with the guidance and resources that have allowed me to press on with my research with the maximum amount of curiosity and determination. I have been lucky here in that the limiting factor to progress has nearly always been the amount of time I am willing to spend on a problem, not the availability of regents or expertise. I would also like to thank Dr. Wing Hang-Tong for her continuing input and suggestions, and her gentle insistence that I should always be looking for the best way to answer my questions with experiments. I am indebted to all of our collaborators, and particularly Dr. Ronald Haller, for providing specimens, reagents, and expertise that have made these studies possible. Finally, I would like to thank my father for cultivating my scientific curiosity since birth.

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INTRODUCTION

Iron serves as a versatile and indispensable enzyme cofactor across all branches of life. Iron-sulfur (Fe-S) clusters are structures consisting of iron atoms coordinated with inorganic sulfur and cysteine sulfur atoms, through which Fe-S clusters covalently attach to proteins. The utility of Fe-S clusters in biological functions lies most prominently in their ability to catalyze the electron transfer reactions that underlie the basic energetic flux of life. Evidence that Fe-S clusters were present in critical electron transfer proteins first came from studies performed in the late 1950’s and early 1960’s by research groups studying the hydrogen-producing bacterium Clostridium \(^1\), the photosynthetic machinery of chloroplasts \(^2\), and succinate dehydrogenase from beef heart mitochondria \(^3\).

Following the discovery and characterization of the various forms and functions of Fe-S cluster-containing proteins, a logical question arose regarding how these clusters were formed within cells. Synthetic Fe-S clusters could be formed \textit{in vitro} with relative ease by utilizing inorganic ferrous iron (Fe\(^{2+}\)), sulfide (HS\(^-\)), reducing agents, and synthetic aryl or alkyl thiolate cluster ligands \(^4\). However, in order to successfully proceed, these synthetic reactions must be set up in non-aqueous solvents, a condition which does not exist within living cells. Consequently, living cells must possess machinery to efficiently assemble Fe-S clusters and protect them from the aqueous intracellular medium. The first concrete evidence for a cellular Fe-S cluster machinery system came from studies of the nitrogen fixation operon (\textit{nif}) of \textit{Azotobacter vinelandii} \(^5\). The \textit{nif} operon contains the genes that encode the subunits of the nitrogen-fixing Fe-S enzyme nitrogenase, as well as several other accessory proteins that are required for the full
catalytic capacity of this enzyme\(^5\). Further work on \(nifS\), one of the accessory genes within the \(nif\) operon, demonstrated that the protein product of this gene had cysteine desulfurase activity which could provide the inorganic sulfide needed for formation of the Fe-S cluster of nitrogenase and other Fe-S enzymes as well\(^6,7\).

Fe-S cluster assembly genes have been found mainly in three distinct operons, which express several different types of Fe-S assembly systems, and are distributed across all kingdoms of life\(^8\). Nitrogen-fixing bacteria contain the \(nif\) operon to allow for the maturation of nitrogenase, while \(Escherichia coli\) contains the \(suf\) and \(isc\) operons to allow for the formation and repair of Fe-S proteins\(^9\). Over 20 genes have been implicated in Fe-S cluster assembly processes in mammals\(^10\); however, the core Fe-S cluster assembly components are remarkably similar to the components of the bacterial \(isc\) Fe-S cluster assembly operon (see Figure 1). The Fe-S cluster assembly process in human cells can be divided into two primary steps involving: (1) Assembly of nascent Fe-S clusters on the Fe-S scaffold protein ISCU, in complex with the cysteine desulfurase NFS1, and the accessory proteins ISD11 and frataxin, and (2) targeted transfer of newly-formed Fe-S clusters from ISCU to recipient apo-proteins, which is facilitated by the chaperones HSC20 and HSC70 and ATP hydrolysis (see Figure 1).
Figure 1: Overview of mammalian Fe-S cluster assembly. Studies in bacteria, yeast, zebrafish, and mammals have all contributed to our understanding of how Fe-S cluster assembly machinery functions in human cells. (A) The scaffold protein ISCU binds to a protein complex consisting of the cysteine desulfurase dimer ISCS/NFS1 and the accessory protein ISD11. Fe-S clusters are formed on ISCU following the provision of ferrous iron (Fe$^{2+}$), probably donated by frataxin, and inorganic sulfide provided by ISCS/NFS1. (B) After Fe-S clusters are formed, they are transferred to recipient apo-proteins through the coordinated action of molecular chaperones including HSC20 and an HSC70 protein (probably HSPA9). ATP hydrolysis by the HSC70 protein is also required before transfer of Fe-S clusters to apo-proteins. (C) Human diseases caused by impaired Fe-S cluster assembly machinery include ISCU Myopathy (ISCU), Friedreich’s ataxia (frataxin), and GLRX5 Sideroblastic Anemia (GLRX5).

The importance of Fe-S cluster assembly processes in human cells has been underscored recently by the discovery of several human diseases that are caused by defects in various components of the Fe-S cluster assembly machinery. The most prevalent disease associated with impaired Fe-S cluster assembly is Friedreich’s Ataxia, caused by low expression of the frataxin protein$^{11}$. Friedreich’s Ataxia is an inherited spinocerebellar neurodegenerative disease in which patients are affected by limb ataxia, weakness and speech problems, and a progressive
hypertrophic cardiomyopathy that often leads to death in early adulthood \(^\text{12}\). More recently a single patient with sideroblastic anemia and tissue iron overload was shown to have a splicing defect in the gene encoding GLRX5 \(^\text{13}\), a protein that has been implicated in the transfer of Fe-S clusters from scaffolds to recipient apo-proteins \(^\text{14}\). Finally, a splicing defect in the \textit{ISCU} gene was recently found to be the cause of a hereditary myopathy affecting families from northern Sweden \(^\text{15,16}\). In this disease, the splicing mutation leads to low ISCU protein expression in patient skeletal muscle, resulting in low muscle Fe-S enzyme activities and life-long exercise intolerance.

In this dissertation thesis, we will explore the interface between inorganic chemistry and human disease by examining novel roles for Fe-S clusters in red blood cell development and in human disease. In the first chapter, we will explore mouse and human tissues to identify settings in which heme biosynthesis depends on Fe-S cluster availability, by virtue of an essential Fe-S cluster cofactor present in the terminal heme biosynthetic enzyme ferrochelatase. In the second chapter we will evaluate the unique tissue specificity of ISCU Myopathy, and will explore novel mechanisms that may pre-dispose muscle tissue to the pathological effects of ISCU deficiency. Finally, we will use global gene expression analysis to examine novel pathways that are activated in ISCU Myopathy patient muscles due to Fe-S cluster deficiency. The results of these studies will have broad implications for the way we think about cellular iron metabolism during normal and pathological human development. Moreover, we can take some of these findings back to the clinic, where they will be utilized for better diagnosis and treatment of diseases caused by impaired Fe-S cluster assembly.
CHAPTER 1

Post-Translational Stability of the Heme Biosynthetic Enzyme Ferrochelatase is Dependent on Iron Availability and Intact Iron-Sulfur Cluster Assembly Machinery

Short Title: Functions of the Fe-S Cluster of Ferrochelatase

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Abstract

Mammalian ferrochelatase, the terminal enzyme in the heme biosynthetic pathway, possesses an iron-sulfur [2Fe-2S] cluster that does not participate in catalysis. We investigated ferrochelatase expression in iron-deficient erythropoietic tissues of mice lacking iron regulatory protein 2 (IRP2), in iron-deficient MEL cells, and in human patients with ISCU myopathy. Ferrochelatase activity and protein levels were dramatically decreased in Irp2−/− spleens, whereas ferrochelatase mRNA levels were increased, demonstrating post-transcriptional regulation of ferrochelatase in vivo. Translation of ferrochelatase mRNA was unchanged in iron-depleted MEL cells, and the stability of mature ferrochelatase protein was also unaffected. However, the stability of newly formed ferrochelatase protein was dramatically decreased during iron deficiency. Ferrochelatase was also severely depleted in muscle biopsies and cultured myoblasts from patients with ISCU myopathy, a disease caused by deficiency of a scaffold protein required for Fe-S cluster assembly. Together, these data suggest that decreased Fe-S cluster availability due to cellular iron depletion or impaired Fe-S cluster assembly causes reduced maturation and stabilization of apo-ferrochelatase, providing a direct link between Fe-S biogenesis and completion of heme biosynthesis. We propose that decreased heme biosynthesis due to impaired Fe-S cluster assembly can contribute to the pathogenesis of diseases caused by defective Fe-S cluster biogenesis.
Introduction

Heme, the iron-containing tetrapyrrole cofactor of hemoproteins, is indispensable for many cellular and organismal metabolic processes due to its ability to confer gas transport and electron transfer functionalities to countless enzymes. In animals, heme biosynthesis begins in the mitochondrion with the conjugation of succinyl-coenzyme A and glycine by δ-aminolevulinic acid synthase (ALAS) to form δ-aminolevulinic acid (ALA)\(^{17}\). ALA is transported into the cytoplasm where it serves as the building block for the tetrapyrrole macrocycle via a series of well characterized reactions\(^{18}\), and the process is concluded in the mitochondrion with insertion of iron into protoporphyrin IX (PPIX) by ferrochelatase to form heme\(^{19}\).

Large amounts of iron and heme are required by developing erythroblasts in order to supply millimolar quantities of hemoglobin in erythrocytes. An iron-responsive element (IRE) present in the 5’ un-translated region (UTR) of the mRNA that encodes erythroid-specific isoform of ALAS (Alas2)\(^ {20}\) allows for binding of iron regulatory proteins 1 and 2 (IRP1/2) during iron deficiency, resulting in decreased synthesis of ALAS2 protein and restricted initiation of heme biosynthesis. In contrast, IRE sequences in the 3’UTR of transferrin receptor 1 (Tfr1) confer protection from nuclease degradation upon IRP binding\(^ {21}\), thus increasing Tfr1 protein expression during iron deficiency. Accordingly, \(Irp2^{−/−}\) erythroblasts express less Tfr1, likely contributing to the low hematocrit and microcytosis observed in these animals despite normal serum transferrin saturation levels\(^ {22,23}\).
*Irp2*<sup>−/−</sup> mice also possess >100-fold elevated levels of erythrocyte PPIX<sup>22</sup>, a condition found in patients affected by erythropoietic protoporphyria (EPP). EPP is characterized by elevated erythrocyte PPIX, resulting in painful cutaneous photosensitivity and, infrequently, hepatic failure caused by biliary occlusions of crystalline protoporphyrin<sup>24</sup>. Whereas elevated erythrocyte PPIX levels in *Irp2*<sup>−/−</sup> mice are associated with the loss of IRP2-mediated translational repression of ALAS2<sup>22</sup>, EPP in humans is usually caused by mutations in the ferrochelatase (*Fech*) gene<sup>25</sup>. EPP patients usually possess less than a third of the normal level of ferrochelatase activity<sup>26</sup>, which most frequently results from inheritance of a mutated *Fech* allele with decreased activity together with a low-expressing normal *Fech* allele<sup>27</sup>.

Unlike the *S. cerevisiae* and *E. coli* enzymes, mammalian ferrochelatase possesses a solvent-exposed [2Fe-2S] cluster<sup>28,29</sup>. Interestingly, replacement of the carboxy-terminal segment of mammalian ferrochelatase containing three of the cluster-ligating cysteines with *S. cerevisiae* ferrochelatase sequence yielded a non-cluster-containing protein that retained some activity<sup>30</sup>. The crystal structure of human ferrochelatase suggests a structural role for the Fe-S cluster by revealing a unique stabilizing bridge formed by the cluster between the three carboxy-terminal cysteines and a fourth internal cysteine<sup>28,31</sup>. Mutations in the four coordinating cysteine residues of recombinant human ferrochelatase resulted in an inactive enzyme<sup>31,32</sup>, consistent with observations in five EPP patients in which such mutations also resulted in inactive enzyme<sup>33</sup>.

Since the Fe-S cluster of ferrochelatase does not participate directly in catalysis, alternative roles have been proposed (reviewed by Dailey et al.<sup>34</sup>). Initial demonstration of decreased ferrochelatase activity in cultured rat hepatocytes treated with S-nitroso-N-acetylpenicillamine<sup>35</sup>, a nitric oxide (NO)-generating compound, prompted two further studies
which showed that the cluster is sensitive to destruction by NO in vitro\textsuperscript{36}, and in cultured cells\textsuperscript{37}. Ferrochelatase activity was also decreased in iron-deprived Cos7 cells expressing human ferrochelatase, but not in cells expressing the non-cluster-containing \textit{E. coli} ferrochelatase\textsuperscript{38}, suggesting that the cluster may participate in the iron-mediated regulation of human ferrochelatase\textsuperscript{38}.

To better understand the role of the Fe-S cluster of ferrochelatase during heme biosynthesis under iron deficient conditions, we investigated ferrochelatase expression in the iron-deficient erythropoietic tissues of \textit{Irp2}\textsuperscript{-/-} mice, in iron-deprived murine erythroleukemia (MEL) cells, and in human patients suffering from ISCU myopathy\textsuperscript{15,16}. Our results demonstrate significant post-transcriptional changes in ferrochelatase expression in \textit{Irp2}\textsuperscript{-/-} splenic erythroblasts. We also found that newly-formed, but not mature, ferrochelatase was affected by iron deficiency in MEL cells and that ferrochelatase was dramatically reduced in skeletal muscle of patients with impaired Fe-S cluster assembly due to ISCU depletion. Together, these results demonstrate the potential for modulation of heme biosynthesis via the availability of newly-formed Fe-S clusters for insertion into the ferrochelatase apo-protein.
Materials and Methods

Animals and Cell Lines

Irp2\textsuperscript{-/-} and Irp2\textsuperscript{-/-}:Irpi\textsuperscript{+/+} animals were generated as described previously\textsuperscript{22}, and were greater than one year of age at the time of analysis. The animals were deeply anesthetized by injection of an isotonic pentobarbital solution containing sodium heparin, and tissues were exsanguinated by cardiac perfusion with phosphate-buffered saline (PBS). Bone marrow was obtained as described previously\textsuperscript{22}. All procedures were approved by the NICHD Animal Care and Use Committee. MEL cells (a gift from Dr. David Bodine) were propagated in HEPES-buffered RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum at 37°C and 5% CO\textsubscript{2}. To induce differentiation, cells were cultured in the presence of 2\% DMSO. Under these conditions, the cells cease to divide within 24h, and hemoglobin accumulation is evident after 48h. All reagents were purchased from Sigma-Aldrich unless otherwise noted.

Splenic Erythroblast Isolation

Splenic cell suspensions were obtained following perfusion with PBS. Incisions in the capsular membrane were made, and the tissue was rubbed between the frosted portion of two microscope slides in PBS. This suspension was passed through a 70\textmu m strainer, and the filtrate was layered onto Ficoll-Paque\textsuperscript{TM} PLUS solution (GE Healthcare). Nucleated cells were separated from mature erythrocytes by centrifuging at 1000 x g for 20min at room temperature. The recovered
supernatant was washed with Hank’s Balanced Salt Solution (HBSS), and erythroblasts were isolated by incubation with streptavidin-coated Dynabeads (Invitrogen) conjugated to biotinylated anti-TER119 antibody\(^{39}\) (eBioscience) using the indirect technique according to the manufacturer’s instructions. Human Fc fragment (Jackson Immunoresearch) was included to block Fc receptor-mediated binding of non-erythroid cells.

**Ferrochelatase Assay**

Ferrochelatase activity was assayed using modifications to the methods of Shepherd *et al.*\(^{40}\), Jones and Jones\(^{41}\), and Li *et al.*\(^{42}\). Samples were sonicated on ice in lysis buffer consisting of 1% sodium cholate dissolved in 50mM Tris-HCl, pH 8.0. After centrifugation at 20,000 x g for 10 min, the supernatants were adjusted to the same total protein concentration following BCA assay (Pierce). Ferrochelatase assay buffer consisted of 20mM Tris-HCl (pH 8), 1% Triton X-100, 1mM lauric acid, 50μM deuteroporphyrin IX (Frontier Scientific, Inc.), and 50μM Zn(II) acetate. A 200-400μg protein sample was added to a UV microcuvette, followed by 10X assay buffer mixture (without deuteroporphyrin IX) for a final concentration of 1X assay buffer in 350μL. Reactions were started by the addition of deuteroporphyrin IX, and the cuvette was placed at 37°C in an Agilent 8453 spectrophotometer. With the UV lamp turned off, visible absorbance spectra were taken in 30 second intervals. Time points between five and ten minutes were used to plot linear kinetic curves. The absorption maximum of the evolving β band (indicative of metal chelation\(^{43}\)) present in the range of the Q bands at 541nm was subtracted from an isobestic point of 524nm present between Q bands IV and III of the un-metallated
porphyrin to yield a first-order kinetic rate. For reaction blanks, 2µM N-methyl-mesoporphyrin IX (Frontier Scientific) was included in the reaction mixture of a duplicate sample, which resulted in complete inhibition of the absorbance increase over time. Data are presented as first-order rates, normalized to control samples as either fold-change or percent of control.

**Hemoglobin Assay**

MEL cell hemoglobin levels were assessed semi-quantitatively by native PAGE separation and electroblotting followed by chromophore-enhanced visualization. Briefly, cell pellets were sonicated in Tris-buffered saline (pH 7.5), and centrifuged for 10min at 20,000 x g, after which the supernatants were centrifuged for 15min at 105,000 x g. The resulting supernatants were adjusted to equivalent protein concentrations, mixed 1:1 with sample buffer (pH 7.5; 100mM Tris-HCl, 15% glycerol, 0.05% bromphenol blue), and 20-40µg of protein was subjected to electrophoresis at 125V using a 4-20% Tris-glycine gel (running buffer was 12mM Tris base, 96mM glycine) until the dye front traveled half way to the bottom of the gel. Proteins in the gel were electroblotted onto a 0.2µm pore-size PVDF filter (Invitrogen) for 1h in alcohol-free transfer buffer (25mM Tris base, 192mM glycine) in a Hoefer TE22 transfer apparatus at a current of 400mA and temperature fixed at 20°C. Hemoglobin visualization was enhanced by incubating the filter in a solution of freshly made and filtered 50mM Tris (pH 7.5), 50mM imidazole, 0.5mg/mL diaminobenzidine, and 0.1% H₂O₂. for 20min in the dark, followed by two rinses in water, and 10min incubation in a solution consisting of 0.5% (w/v) CuSO₄ made fresh in 50mM Tris-HCl, pH 7.5. The filter was washed, dried overnight in the dark, and digitized the
next morning. Mouse erythrocyte lysate was used as a positive control for hemoglobin in initial experiments. A prominent protein band observed after further staining of the filters with 0.1% Ponceau-S was used as a protein loading control.

**Western Blots**

SDS-PAGE and western blotting were performed as described previously using 1.5mm 10% pre-cast bis-Tris gels (Invitrogen), except for ISCU, which was resolved using large-format 15% Tris-glycine gels. Rabbit polyclonal anti-ferrochelatase serum was a kind gift from Dr. Harry Dailey. Mouse monoclonal anti-SOD2 was from Abcam. Anti-mitochondrial aconitase and ISCU rabbit polyclonal sera were raised against synthetic peptide fragments. Mouse monoclonal anti-PPOX was from Abnova. ALAS2 antibody was affinity purified using a modified surface affinity purification technique (manuscript in preparation). The ALAS2 band, which runs at an aberrantly low apparent molecular weight (MW) in bis-Tris gels, was confirmed to be of the correct MW when run in Tris-glycine gels.

**Northern Blots and qRT-PCR Analysis**

RNA was extracted using Trizol (Invitrogen), and further purified using the RNeasy™ kit (Qiagen) cleanup protocol. Northern blots were performed as reported previously. dsDNA probes were made using α-32P-deoxycytosine triphosphate (Perkin Elmer) and the Megaprime labeling kit (GE Healthcare). Probe templates were generated by PCR using a MEL cell cDNA
library. Primers were as follows: Probe for both mouse and human ferrochelatase: 5’-ATGGAGAGAGATGGACTAGAGAGGGCC-3’ and 5’-CGCTCTTCTGTGTTCTCAGCTCCACA-3’; mouse β-globin: 5’-GTTGTGTTGACTTGCAACCTCAGA-3’ and 5’-CATTTCCCCACAATTGACAGTTTT-3’.

For ALAS2, a probe template was obtained by restriction digestion of a pHIS-Parallel plasmid containing mouse ALAS2 protein coding sequence. qRT-PCR was performed using SYBR® Green (Applied Biosystems) according to the manufacturers instructions, following reverse transcription of total RNA into cDNA (Applied Biosystems). qRT-PCR primers were as follows: mouse ferrochelatase 2.2 + 2.9kb transcripts: 5’-CCTCATCCAGTGCTTTGCAGA-3’ and 5’-GCAGGGGATGGGCAGAAAAC-3’; mouse ferrochelatase 2.9kb transcript only: 5’-CGCCTTCATTTGAGTCCACAGT-3’ and 5’-GGCACTGGAAGGTTCCTTGAGGTTTCCCTTGG-3’. Relative transcript abundance was calculated using the 2^ΔΔCt method\(^45\), with GAPDH as the internal control. Correct qRT-PCR product size was verified by agarose gel electrophoresis.

**Aconitase In-Gel Assay and Electrophoretic Mobility Shift Assay (EMSA)**

Aconitase was assayed using a coupled assay following native PAGE separation, as described previously\(^46\). IRP-IRE binding activity was determined by EMSA using a \(^32\)P-labeled ferritin IRE probe, as described previously\(^46\).

**Metabolic Labeling and Pulse-Chase**
The core protocol for metabolic labeling of MEL cells used in this study is as follows. Cells were washed once in labeling medium (RPMI1640 without L-cysteine and L-methionine), and incubated in labeling medium for 1h, followed by metabolic labeling with the EXPRESS $^{35}$S-cysteine and $^{35}$S-methionine reagent (1175Ci/mmol; Perkin Elmer) for 10-60min. Subsequently, cells were washed with complete RPMI medium and frozen immediately, or washed twice and chased in treatment medium for various time points. Cell pellets were lysed in Tris-buffered saline with 1% Triton X-100, centrifuged at 20,000 x g for 10min, and adjusted to equal total protein concentration. Immunoprecipitation was carried out essentially as described previously$^{47}$, with 750µg of total protein and 1.5µL of ferrochelatase antiserum used per sample. Wash and binding buffer consisted of 1% NP-40 and 0.5% deoxycholate in PBS. Immunoprecipitated radio-labeled ferrochelatase was visualized by SDS-PAGE in 10% bis-Tris gels with MOPS-SDS running buffer (Invitrogen), followed by autoradiography with Kodak MR single emulsion films.

**Tissue Biopsies and Myoblast Cultures**

Control and ISCU myopathy patient skeletal muscle was obtained and prepared as described previously$^{15}$. Written consent was given by all individuals involved in the study in accordance with the Institutional Review Board for Human Studies of the University of Texas, Southwestern Medical School. Primary myoblast cell lines, a generous gift from Dr. Eric Shoubridge, were cultured on BD Matrigel™-coated flasks as described previously$^{48}$, except that Lonza SKBM-2 culture medium was used. Terminal differentiation of myoblasts into myotubes was achieved by
incubation of the cultures for four days in DMEM containing 2% heat-inactivated horse serum (Invitrogen).
Results

Post-transcriptional alteration of ferrochelatase expression in iron-deficient erythropoietic tissues of $Irp2^{-/-}$ mice.

Depleted cellular iron stores and microcytic anemia have been correlated with reduced TfR1 protein expression levels in the developing erythroblasts of $Irp2^{-/-}$ mice. In order to investigate the expression of ferrochelatase in functionally iron-deficient erythroid tissue, ferrochelatase activity, protein, and mRNA levels were measured in bone marrow and spleens of wild type ($Irp1^{+/+}:Irp2^{+/+}$) and $Irp2^{-/-}$ ($Irp1^{+/+}:Irp2^{-/-}$) mice. Ferrochelatase activity, protein levels, and mRNA abundance were all decreased in $Irp2^{-/-}$ bone marrow aspirates as compared to wild type animals (Figure 1A-C); however, while ferrochelatase activity and protein levels were also decreased in $Irp2^{-/-}$ spleens (Figure 1D,E), ferrochelatase mRNA levels were increased (Figure 1F). Northern blots of $\beta$-globin in bone marrow aspirates demonstrated decreased $\beta$-globin mRNA levels in $Irp2^{-/-}$ mice and in $Irp2^{-/-}$ mice lacking one allele of $Irp1$ ($Irp1^{+/+}:Irp2^{-/-}$; Figure 1G), whereas spleen $\beta$-globin mRNA levels were elevated in $Irp2^{-/-}$ and $Irp1^{+/+}:Irp2^{-/-}$ mice (Figure 1H). Splenic expression levels of the erythroid-specific transcription factors $Gata1$ and $Hmgb3$ were also increased, as measured by qRT-PCR (data not shown). These data suggest that erythropoietic activity shifted somewhat from the bone marrow to the spleen in $Irp2^{-/-}$ and $Irp1^{+/+}:Irp2^{-/-}$ animals, which might explain the changes in ferrochelatase mRNA levels observed in bone marrow and spleens of $Irp2^{-/-}$ mice.
To eliminate the effect of differences in splenic cellularity on our analysis of ferrochelatase expression, TER119+ erythroid cells were isolated from animals of the three genotypes, and ferrochelatase mRNA and protein levels in each sample were evaluated by qRT-PCR and western blot. Ferrochelatase protein was significantly decreased in splenic TER119+ erythroid cells from $Irp2^{-/-}$ and $Irp1^{+/+}:Irp2^{-/-}$ animals (Figure 1I), and despite some variation in protein and mRNA levels, the calculated protein:mRNA ratio was significantly decreased in the $Irp2^{-/-}$ and $Irp1^{+/+}:Irp2^{-/-}$ samples (Figure 1J). Notably, mild decreases in ferrochelatase activity and protein levels were also observed in the kidneys and livers of $Irp1^{+/+}:Irp2^{-/-}$ mice in the absence of measurable changes in ferrochelatase mRNA levels, whereas ferrochelatase activity and protein levels were unchanged in whole brain and heart extracts (data not shown).

**Post-transcriptional regulation of ferrochelatase in MEL cells during iron depletion and oxidative stress**

To allow for further investigation of the post-transcriptional regulation of ferrochelatase in developing erythroid cells, we established a model of iron-limited erythroid differentiation using MEL cells. Treatment of MEL cells with 2% DMSO induces rapid progression of MEL cells from a stage resembling late erythroid colony-forming units (CFU-E) to a stage resembling basophilic and orthochromatophilic erythroblasts in the erythroid lineage\textsuperscript{49}, where globin expression and hemoglobin formation are observed, along with increased expression of ferrochelatase mRNA and protein\textsuperscript{50}. Treatment of differentiating MEL cells concomitantly with the iron chelator desferrioxamine (DFO) caused a sustained increase in IRE binding activity of
IRP1 and IRP2 (Figure 2B), whereas the differentiation-induced increase in activity of the [4Fe-4S] cluster-containing mitochondrial and cytosolic aconitases was attenuated (Figure 2C), consistent with cellular iron depletion. Induction of *Alas2* mRNA expression was observed during differentiation of MEL cells under both normal and iron-depleted conditions (Figure 2D). However, ALAS2 protein levels remained repressed in iron-deficient MEL cells (Figure 2E), presumably due to increased IRP-mediated translational repression. Finally, hemoglobin formation was attenuated in iron-depleted MEL cells (Figure 2F), an effect that was likely due to a combination of decreased iron availability and repressed ALAS2 protein induction\(^5\).

As reported previously\(^5\), expression of both the short (2.2kb) and long (2.9kb) isoforms of ferrochelatase mRNA transcripts increased during DMSO-induced differentiation of MEL cells (Figure 3A), leading to increased ferrochelatase activity and protein levels (Figure 3A,B). In contrast, ferrochelatase activity and protein levels were attenuated in iron-depleted differentiating MEL cells, despite comparable increases in ferrochelatase mRNA (Figure 3C,D). In summary, differentiation of MEL cells under iron-deficient conditions faithfully reproduces the post-transcriptional decrease of ferrochelatase protein observed in iron-deficient *Irp2\(^-\)/-* erythroblasts shown above, and as reported previously in DFO-treated K562 cells\(^3\), providing us with a system to further evaluate how ferrochelatase protein and activity decrease during iron deficiency.

In contrast to the effects of iron depletion, we observed greater abundance and activity of ferrochelatase in MEL cells cultured in a 6% O\(_2\) vs. a 21% O\(_2\) atmosphere for several days, followed by induction of differentiation by treatment with DMSO (Figure 4B,C). As ferrochelatase mRNA levels were not increased in cells grown in 6% O\(_2\) as compared to cells
grown at 21% O$_2$ (Figure 4D) we surmise that the ferrochelatase holo-protein is stabilized under reduced cellular oxygen concentrations *in vivo*, consistent with the original report of reduced stability of the purified enzyme under aerobic conditions *in vitro*\textsuperscript{29}. The increased ferrochelatase activity and protein levels in cells cultured at 6% O$_2$ was associated with increased ALAS2 protein expression (Figure 4E) and enhanced hemoglobin production (Figure 4F), demonstrating that hemoglobinization of MEL cells is more rapid at reduced oxygen levels. To further investigate the sensitivity of ferrochelatase to oxidative damage *in vivo*, we treated MEL cells with the redox-cycling mitochondrial pro-oxidant menadione\textsuperscript{52}. Brief treatment of MEL cells with a range of menadione concentrations resulted in a rapid dose-dependent depletion of total cellular ferrochelatase protein levels within three hours (Figure 4G), whereas the abundance of several other mitochondrial enzymes including protoporphyrinogen oxidase (PPOX), mitochondrial aconitase (mACO), and superoxide dismutase 2 (SOD2) was not affected within this time period. These data suggest that mature Fe-S cluster-containing ferrochelatase is rapidly degraded during conditions of mitochondrial oxidative stress.

**Stability of newly-formed, but not mature ferrochelatase depends on iron availability.**

In order to better understand the mechanism by which depletion of cellular iron leads to decreased levels of ferrochelatase protein, we performed a series of metabolic labeling experiments to assess the fate of ferrochelatase in MEL cells under the iron-limited differentiation conditions described above. In the first experiment, the cells were labeled with $^{35}$S-cysteine and $^{35}$S-methionine for a brief (10 minutes) period after a 24h treatment of
differentiating MEL cells with DFO. These conditions were used to estimate the “instantaneous” synthesis rate of the ferrochelatase apo-protein under iron-limited conditions. Although total cellular ferrochelatase levels were reproducibly reduced in iron-depleted cells, the rate of synthesis of newly labeled ferrochelatase polypeptides in iron-depleted cells during the 10 minute labeling period was not different from control cells (Figure 5A).

In the subsequent experiment, MEL cells were metabolically labeled for one hour, and were then incubated in normal medium for three hours in order to allow for the newly labeled ferrochelatase proteins to completely fold and obtain Fe-S clusters prior to the onset of iron deficiency. Subsequently, the cells were treated with DMSO ±DFO for 24 hours, and were then harvested and analyzed for total and radio-labeled ferrochelatase as in the previous experiment. Results of this experiment (Figure 5B) demonstrate that the ferrochelatase polypeptides that were allowed to mature fully prior to the onset of iron deficiency were not susceptible to degradation, despite the fact that total cellular ferrochelatase levels were again diminished in the DFO-treated cells.

In the final experiment, MEL cells were first treated with DMSO ±DFO for 24h, followed by metabolic labeling for 40min. Subsequently, the cells were incubated in normal medium for various periods of time followed by assessment of the remaining radio-labeled ferrochelatase (Figure 5C). We found that the half-life of newly formed ferrochelatase was dramatically reduced in the iron depleted cells (Figure 5C,D), in which newly-formed ferrochelatase polypeptides had a calculated half-life of less than 1h. In contrast, ferrochelatase showed a calculated half-life of approximately 35h in control cells. Together, these data suggest
that newly-formed ferrochelatase protein that has not fully folded into the mature form within the mitochondrion is highly susceptible to degradation under iron-deficient conditions.

**Disruption of Fe-S cluster assembly results in diminished cellular ferrochelatase levels.**

The above data suggested that impairment of mitochondrial Fe-S cluster assembly by iron depletion could result in enhanced degradation of newly-formed ferrochelatase polypeptides by limiting the availability of free [2Fe-2S] clusters for insertion into the apo-protein. To test the dependence of ferrochelatase stability on availability of newly-formed Fe-S clusters, we measured ferrochelatase levels in muscle biopsies and cultured myoblasts from patients with ISCU myopathy, a disease characterized by impaired Fe-S cluster assembly in muscle tissue due to deficiency of the Fe-S cluster scaffold protein ISCU\(^{15,16}\) (Figure 6A). Ferrochelatase protein levels were dramatically depleted in ISCU patient muscle biopsies, as compared to three control biopsies and one biopsy obtained from a patient with an unrelated mitochondrial myopathy (Figure 6B). As shown previously\(^{15}\), levels of [4Fe-4S]-containing mitochondrial aconitase (mACO) were also depleted in the ISCU myopathy patients (Figure 6B).

To further investigate ferrochelatase expression levels during ISCU depletion, a differentiation time-course experiment was performed using cultured myotubes isolated from an ISCU myopathy patient and control myotubes isolated from a normal individual. Both ISCU and ferrochelatase protein levels increased significantly during the course of differentiation of the myoblasts into myotubes (Figure 6C). However, marked ISCU depletion was observed in the patient cultures, which was accompanied by decreased ferrochelatase protein levels relative to
the control at every respective time point (Figure 6C). In contrast, ferrochelatase mRNA levels, measured in control and patient myotube cultures at 0 and 5 days of differentiation by northern blot (Figure 6D) and qPCR (data not shown), did not reflect the dramatic decrease in ferrochelatase protein levels observed in the patient tissues (Figure 6B) and myotube cultures (Figure 6C), confirming post-transcriptional regulation of ferrochelatase during conditions of impaired Fe-S cluster assembly in the ISCU myopathy patients.
Discussion

Since the significant discovery that mammalian ferrochelatase is an Fe-S cluster-containing protein\textsuperscript{29}, there has been continued speculation on possible biological functions of the cluster, which has been renewed following biochemical and structural reports demonstrating that the cluster does not participate directly in enzymatic catalysis\textsuperscript{28,30}. In this study we report significant post-transcriptional depletion of ferrochelatase during iron-deficient erythropoiesis in \textit{Irp2\textsuperscript{−/−}} mouse erythroid tissue, during iron-limited erythroid differentiation of MEL cells \textit{in vitro}, and during conditions of impaired \textit{de-novo} Fe-S cluster assembly in human patients affected by ISCU myopathy. Together these findings suggest that ferrochelatase expression is significantly influenced by the availability of newly-formed Fe-S clusters, the biogenesis of which is reliant upon the continued availability of iron as well as functional Fe-S cluster assembly machinery. Recently, a pool of genes that co-express with the heme biosynthetic enzymes was reported\textsuperscript{53}, and several of these genes have been implicated (\textit{C1orf69}\textsuperscript{54}), or are known to participate directly (\textit{GLRX5}\textsuperscript{55}, \textit{ISCA1} and \textit{ISCA2}\textsuperscript{56}) in Fe-S cluster assembly. These observations, along with the results presented here, provide further evidence for an inextricable link between Fe-S cluster assembly and heme biosynthesis.

**Decreased ferrochelatase expression during iron-limited erythropoiesis in IRP2\textsuperscript{−/−} mice**

Ferrochelatase is essential for heme biosynthesis in all mammalian cells, and ferrochelatase expression is robustly up-regulated in developing erythroid cells by transcriptional
activation via a single promoter containing Sp1, NF-E2 and GATA elements \(^5\). In erythroid cells, iron limitation decreases heme biosynthesis via IRP-mediated translational repression of ALAS2 due to the presence of an IRE in its 5’UTR\(^{20}\), and loss of this repression in \(Irp2^{-/-}\) mice undoubtedly contributes to the >100-fold increase in PPIX observed within their erythrocytes\(^{22}\).

Here we report an additional alteration in the heme biosynthetic pathway in \(Irp2^{-/-}\) mice, namely, post-transcriptional depletion of ferrochelatase protein levels in developing \(Irp2^{-/-}\) splenic erythroblasts. Despite the significantly increased levels of ferrochelatase, β-globin, and other erythroid-specific mRNA transcripts observed in \(Irp2^{-/-}\) spleens, likely a consequence of enhanced extramedullary hematopoiesis, ferrochelatase activity and protein levels were significantly decreased (Figure 1). Erythropoietic cells in \(Irp2^{-/-}\) mice are considered to be constitutively iron-deficient due to multiple factors including decreased expression of TfR1\(^{22,23}\).

We hypothesize that iron deficiency within developing \(Irp2^{-/-}\) erythroblasts limits the availability of newly-formed Fe-S clusters for insertion into ferrochelatase apo-proteins. Further, we predict that the ferrochelatase apo-protein is rapidly degraded by mitochondrial proteases when it cannot obtain an Fe-S cluster, since the cluster-ligating carboxy-terminal region of the protein is important for folding and dimerization of the mature enzyme\(^{28,32}\), and mutations in this region result in EPP in humans\(^{33}\).

**Post-translational decrease in ferrochelatase protein during iron-limited erythropoiesis and oxidative stress in MEL cells**
To more closely examine the effect of ferrochelatase during cellular iron deficiency we employed differentiating MEL cells grown under mild iron-deficient conditions, characterized by activation of IRPs, repression of ALAS2 protein expression, and delayed hemoglobin formation. The expected increase in ferrochelatase protein levels during differentiation was strongly attenuated under these conditions even though ferrochelatase mRNA levels were elevated. Cellular iron deficiency might alter ferrochelatase levels either by repressing the translation of ferrochelatase polypeptides, by reducing the *de-novo* formation of mature cluster-containing holo-protein, or by decreasing the stability of the mature holo-protein. Using metabolic labeling and pulse-chase experiments, we found no alteration in the instantaneous synthesis rate of ferrochelatase during iron deficiency; however, the apparent half-life of the newly formed ferrochelatase was dramatically reduced from ~35h in normal cells to <1h in iron-deficient cells. Perhaps most importantly, no change in the abundance of $^{35}$S-labeled mature ferrochelatase protein was observed following iron depletion. Together, we interpret these results to suggest that mature mitochondrial [2Fe-2S] cluster-containing holo-ferrochelatase is not susceptible to degradation during iron deficiency, while newly-formed apo-ferrochelatase is rapidly degraded in the absence of available iron.

In contrast to cellular iron deficiency, where we observed no depletion of mature ferrochelatase, we did observe very rapid (within 3h) decreases in total cellular ferrochelatase levels following menadione treatment, which cannot be explained by impaired maturation of the apo-protein because of the short time-frame of the experiment. Together with our finding of increased ferrochelatase stability in MEL cells grown at reduced O$_2$ levels, these data suggest that the oxidative modification of mature ferrochelatase, likely involving alteration or destruction
of the Fe-S cluster, leads to rapid degradation of the protein. These results are consistent with the previous report on decreased stability of ferrochelatase under aerobic conditions in vitro\textsuperscript{29}, and other reports concerning the sensitivity of ferrochelatase to nitric oxide\textsuperscript{35-37}.

**Ferrochelatase protein expression depends on Fe-S cluster availability**

It is logical to predict that the diminished maturation of ferrochelatase polypeptides in the mitochondria of iron-depleted cells is due to impaired de-novo formation of [2Fe-2S] clusters by the mitochondrial Fe-S cluster assembly machinery due to lack of available iron ions. Under this prediction, one would expect a similar decrease in cellular ferrochelatase levels following impairment of the Fe-S assembly machinery by means other than iron depletion. To test this hypothesis we measured cellular ferrochelatase levels in muscle biopsies and cultured myoblasts obtained from Swedish patients with ISCU myopathy, a mitochondrial myopathy with exercise intolerance that was recently shown to be a result of deficiency of the Fe-S cluster scaffold protein ISCU\textsuperscript{15,16}. In these patients, the activity and abundance of several Fe-S-containing proteins, including aconitase and succinate dehydrogenase, is greatly reduced\textsuperscript{58}, leading to impaired muscle oxidative phosphorylation, lactic acidosis, and episodes of acute rhabdomyolysis and myoglobinuria\textsuperscript{58,59}.

Here, we report a dramatic reduction in ferrochelatase protein levels in ISCU myopathy patients, demonstrating that impaired Fe-S cluster assembly in the absence of iron depletion also decreases ferrochelatase expression post-transcriptionally. We predict that a similar mechanism can explain our observations during both cellular iron deficiency and during impaired Fe-S
cluster assembly. Decreased availability of newly-formed Fe-S clusters caused either by a lack of available iron, or by impairment of the Fe-S cluster assembly machinery results in impaired maturation of apo-ferrochelatase and subsequent rapid degradation by unknown proteases (Figure 7). In contrast, alteration or destruction of the Fe-S cluster of holo-ferrochelatase during oxidative stress or in the presence of nitric oxide rapidly leads to degradation of the mature pool of ferrochelatase, which is otherwise resistant to iron depletion and impairment of Fe-S cluster assembly. Thus, the capacity for cellular heme biosynthesis could be altered by decreased ferrochelatase protein levels brought on by iron deficiency, defects in Fe-S cluster assembly machinery, or oxidative conditions causing Fe-S cluster degradation.

Our findings of decreased ferrochelatase abundance during impaired Fe-S assembly in ISCU myopathy are consistent with recent findings in the mouse model of Friedrich’s ataxia. Decreases in the abundance of mitochondrial Fe-S cluster-containing proteins including ferrochelatase were observed in cardiac tissue of mice lacking frataxin, the deficiency of which is known to result in impaired Fe-S cluster assembly, accompanied by cerebellar ataxia and cardiomyopathy (reviewed elsewhere). These authors also reported increases in the mitochondrial proteases ClpP and Lon in affected mouse cardiac tissue, suggesting a pathway by which accumulating Fe-S cluster-deficient ferrochelatase and other apo-proteins might be degraded. It will be interesting to determine the exact pathway for proteolytic degradation of immature apo-ferrochelatase in the mitochondrion, though this is currently a difficult question to address due to the lack of specific inhibitors of the mammalian mitochondrial protein degradation machinery.
The deficiency of ferrochelatase in ISCU myopathy might help to explain the decreased cytochrome oxidase (Complex IV) activity observed in some ISCU myopathy patients\textsuperscript{63}. In fact, the most dramatic decrease in cytochrome oxidase activity was in two brothers with a more severe compound-heterozygotic form of ISCU myopathy\textsuperscript{63}. While cytochrome oxidase does not contain iron-sulfur clusters, it does contain two heme cofactors essential for electron transport. We propose that the substantial loss of ferrochelatase due to impaired Fe-S cluster assembly in these patients may lead to an inadequate availability of heme for assembly into newly-formed apo-hemoproteins such as cytochrome oxidase. Thus, the loss of ferrochelatase could clearly contribute to the oxidative defects observed in the mitochondrial respiratory chain of these patients.

In summary, we have identified several \textit{in vivo} situations in which robust post-transcriptional decrease in ferrochelatase levels occurs in conjunction with cellular iron deficiency, defective Fe-S cluster assembly, or oxidative stress. Iron deficiency in developing \textit{Irp2}\textsuperscript{−/−} red cells or in MEL cells leads to decreased maturation of Fe-S cluster-containing ferrochelatase, while deficiency in the Fe-S cluster assembly machinery in the muscles of ISCU myopathy patients also leads to substantial depletion of ferrochelatase, perhaps contributing to the pathogenesis of this disease. Together, these results provide concrete examples of an inextricable link between the essential processes of Fe-S cluster assembly and heme biosynthesis.

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Authorship Contributions

D.R.C. designed and performed experiments, analyzed data, and prepared the manuscript. M.C.G. performed experiments. R.G.H. provided vital reagents. W.H.T and T.A.R oversaw the study and designed experiments, and T.A.R. also assisted in manuscript preparation.

Conflict of Interest Disclosure

The authors declare no conflict of interest.
Figure 1: Post-transcriptional reduction of ferrochelatase (FECH) activity and protein levels in erythropoietic tissues of IRP2−/− mice. (A,D) Ferrochelatase activity in bone marrow aspirates and whole spleens of Irp1+/+::Irp2−/− mice was significantly decreased as compared to Irp1+/+::Irp2+/+ (wild type) animals. Error bars are standard deviation (n=4 animals per genotype). (B,E) Ferrochelatase protein levels in bone marrow and spleen were also decreased in Irp1+/+::Irp2−/− animals. Each lane from the western blot represents whole-tissue protein extracts pooled from two animals. The filter was re-probed for superoxide dismutase 2 (SOD2) as a loading control for mitochondrial matrix protein. (C,F) Ferrochelatase mRNA levels were increased in the spleens of Irp1+/+::Irp2−/− mice. Messenger RNA levels were measured by northern blot using a probe specific for both the 2.2kb and 2.9kb FECH transcripts; each lane represents RNA pooled equally from two animals. The 18S ribosomal band was visualized to assess equal loading. Results were confirmed by qRT-PCR (data not shown). (G,H) To estimate the tissue distribution of erythroid cells, β-globin mRNA levels in bone marrow aspirates and spleens from Irp1+/+::Irp2+/+, Irp1+/+::Irp2−/−, and Irp1−/−::Irp2−/− mice were assessed by northern blot. (I) Ferrochelatase protein abundance in splenic TER119+ erythroid cells mice was measured by western blot. Ferrochelatase mRNA transcript levels (J; filled bars) were measured by qRT-PCR using primers specific for both the 2.2kb and 2.9kb FECH transcripts; data are normalized to Irp1+/+::Irp2+/+ (wild type) mRNA levels. Relative protein abundance was calculated by densitometry, and individual protein abundance values were normalized to the respective mRNA levels measured in the same sample (J; open bars). Data in A, D and J were analyzed by two-tailed Student’s T-test; *P<0.05; **P<0.01.
Figure 2: Iron-limited erythroid differentiation of MEL cells. (A) Timeline depicting the experimental time-course of DMSO-induced erythroid differentiation of MEL cells under normal conditions or iron-deficient conditions induced by the iron chelator desferrioxamine (DFO). (B) Electrophoretic mobility shift assay of MEL cell protein extracts using a $^{32}$P-labeled ferritin IRE probe showed activation of IRP1 and IRP2 following treatment with DFO. (C) Aconitase activity gel demonstrating a time-dependent increase in mitochondrial (m-) and cytosolic (c-) aconitase activity levels over the course of differentiation, which was attenuated in DFO-treated cultures. (D) Erythroid aminolevulinic acid synthase 2 (ALAS2) mRNA was induced during differentiation in the presence or absence of DFO. However, ALAS2 protein expression (E) was repressed in DFO-treated cells. Sample loading was assessed by re-probing for actin mRNA and protein, respectively. (F) Mature (heme-containing) hemoglobin (Hb) formation was repressed in differentiating MEL cells treated with DFO. Hemoglobin was measured by a modified diaminobenzidine procedure after separation of total cellular protein (40µg) by native PAGE followed by transfer to PVDF filters (see Materials and Methods).
Figure 3: Post-transcriptional reduction of ferrochelatase (FECH) in MEL cells during iron-limited differentiation. (A,C) Induction of ferrochelatase enzyme activity (red line), but not mRNA levels (blue, green lines) was repressed during differentiation in the presence of DFO. Ferrochelatase mRNA levels were measured by qRT-PCR as described in Materials and Methods. mRNA transcript and enzyme activity levels are normalized to T=0 samples. (B,D) Induction of ferrochelatase protein levels during differentiation was attenuated in cells co-treated with DFO, despite increased ferrochelatase mRNA levels. Ferrochelatase protein levels were measured by western blot, and the filter was re-probed for superoxide dismutase 2 (SOD2) as a loading control for mitochondrial matrix proteins.
Figure 4: Ferrochelatase is destabilized by oxygen and mitochondrial oxidative stress. (A) Cells were maintained in normal (21%) or reduced (6%) O$_2$ atmosphere, followed by differentiation by DMSO treatment. Ferrochelatase activity (B) and protein levels (C) were increased in differentiating MEL cells maintained at 6% O$_2$ relative to 21% O$_2$ cultures. However, ferrochelatase mRNA levels (D) in 6% O$_2$ cultures were not greater than those in 21% O$_2$ cultures. (E) ALAS2 protein expression was also elevated in cells cultured in 6% O$_2$ relative to 21% O$_2$ cultures. (F) Hemoglobinization was accelerated in differentiating cells cultured in 6% O$_2$. (20µg of total protein was loaded) (G) Menadione-induced mitochondrial oxidative stress caused rapid and specific depletion of total cellular ferrochelatase protein levels, while protoporphyrinogen oxidase (PPOX), mitochondrial aconitase (mACO), and SOD2 protein levels were not appreciably altered. Error bars in (B) represent the range of two experimental replicates.
Figure 5

Figure 5: Newly-formed, but not mature ferrochelatase (FECH) protein is susceptible to regulation by iron availability. (A) To test for changes in the instantaneous synthesis rate of ferrochelatase protein under iron-depleted conditions, MEL cells were differentiated for 24h in the presence or absence of DFO, and harvested immediately following a rapid 10min pulse with $^{35}$S-Cys and $^{35}$S-Met. Radiolabeled ferrochelatase was visualized by autoradiography following immunoprecipitation and SDS-PAGE (top panel), while total protein levels were measured by western blot (bottom panel). (B) The effect of iron limitation on mature, Fe-S cluster-containing ferrochelatase was assessed by metabolic labeling of cells for 1h with $^{35}$S-Cys and $^{35}$S-Met 4h prior to the onset of 24h of differentiation and DFO treatment. Following 24h incubation, cells were harvested and analyzed for radiolabeled and total protein levels as in (A). (C) A pulse-chase experiment was performed to follow the fate of newly-formed ferrochelatase protein during normal and iron-limited growth conditions. Following 24h differentiation in the presence or absence of DFO, cells were pulsed for 40min with $^{35}$S-Cys and $^{35}$S-Met, followed by incubation for various periods of time in differentiation medium with or without DFO. A representative autoradiogram is shown; the results of two experiments were quantified and plotted in (D).
Figure 6: Disruption of Fe-S assembly in ISCU myopathy causes depletion of ferrochelatase. (A) As reported previously, total ISCU protein levels were depleted in vastus lateralis muscle biopsies taken from Swedish patients with ISCU myopathy (lanes designated as P1-P3), as compared to three healthy controls (designated C1-C3), and a patient with an unrelated mitochondrial myopathy (designated as M1). A prominent protein band observed following Ponceau-S staining of the filter served as the loading control. (B) Ferrochelatase protein levels were also greatly decreased in the ISCU myopathy biopsies, as were levels of the Fe-S cluster-containing enzyme mitochondrial aconitase. (C) Primary myotube cultures obtained from an ISCU myopathy patient as well as from a healthy individual (control) were terminally differentiated by growth in low serum conditions (see materials and methods). Although ferrochelatase and ISCU levels increased in both control and patient cultures during the course of the experiment, the relative level of both proteins was diminished in the patient cultures as compared to the control at every given time point. (D) Little difference in ferrochelatase mRNA levels was seen in control and patient myotube cultures at zero and five days of differentiation, as assessed by northern blot.
Figure 7: The impact of cellular iron deficiency, impaired Fe-S biosynthesis, and mitochondrial oxidative stress on ferrochelatase activity and protein levels. (A) A schematic representation of the synthesis, mitochondrial translocation, and maturation of ferrochelatase polypeptides under normal conditions. Ferrochelatase protein is synthesized on cytosolic ribosomes and translocated into the mitochondrion where its signal peptide is cleaved. Complete folding and maturation of ferrochelatase requires the provision of a newly-formed Fe-S cluster, which is supplied by the Fe-S cluster assembly machinery. After folding and insertion of the cluster, ferrochelatase can catalyze the final step in the heme biosynthetic pathway, which is the insertion of ferrous iron into protoporphyrin IX. (B) Under conditions of cellular iron depletion, de-novo Fe-S cluster assembly in the mitochondrion is halted due to the lack of available iron ions, and newly-imported apo-ferrochelatase accumulates and is rapidly degraded within the mitochondrion. (C) Similarly, if mitochondrial Fe-S assembly is disrupted in the absence of cellular iron depletion, as in ISCU myopathy, apo-ferrochelatase fails to obtain Fe-S clusters and is rapidly degraded. (D) Under conditions of mitochondrial oxidative stress, mature Fe-S cluster-containing ferrochelatase is rapidly destabilized. Degradation is likely initiated by chemical modification or disassembly of the Fe-S cluster, resulting in a conformational change and degradation of the ferrochelatase polypeptide.
CHAPTER 2

Abnormal splicing of ISCU, low constitutive protein expression, and oxidative stress contribute to tissue specificity of human ISCU myopathy

Short Title: Tissue Specificity and Mechanisms in ISCU Myopathy

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Abstract

Iron-sulfur (Fe-S) clusters are formed on the scaffold protein ISCU, which provides Fe-S clusters for numerous enzymes and respiratory chain complexes I-III. We observed that ISCU levels in muscle from patients with ISCU myopathy were nearly undetectable, and ISCU levels were also unexpectedly low in healthy human and mouse skeletal muscle tissue. Northern blots identified two predominant patient-specific ISCU mRNAs possessing 100bp or 1073bp of intronic sequence, both of which encoded a truncated and short-lived ISCU protein. Among four tested patient cell types, skeletal muscle biopsies showed the lowest levels of normal ISCU mRNA, and the lowest ISCU protein levels. Over-expression of the muscle-specific transcription factor MyoD1 decreased the amount of normal ISCU mRNA expressed in patient myoblasts, suggesting that the process of terminal muscle differentiation enhances aberrant ISCU mRNA splicing. Mitochondrial and cytosolic aconitases in patient cells were slow to recover from exposure to oxidative stress, an effect that could be rescued by mitochondrial ISCU expression. Surprisingly, cellular ISCU protein levels were rapidly diminished by exposure to H₂O₂ and high O₂, but were protected by pre-treatment with the antioxidant sodium ascorbate. We propose that increased production of reactive oxygen species production during exercise contributes to the extraordinarily low ISCU protein levels that we observed in muscle tissue and to the phenotype of exercise-induced rhabdomyolysis ISCU myopathy patients. The phenomenon of abnormal splicing, which is enhanced by differentiation, likely synergizes with destabilization of ISCU by reactive oxygen species and low constitutive expression levels in skeletal muscle to decrease ISCU expression to pathologically low levels in patient skeletal muscles.
Introduction

ISCU Myopathy, also known as “Myopathy with deficiency of succinate dehydrogenase and aconitase”, is a rare autosomal recessive disease found in individuals of northern Swedish descent. The disease is characterized by life-long exercise intolerance, and trivial bouts of exercise can cause tachycardia and palpitations, dyspnea, muscle fatigue, and lactic acidosis \(^{59,64,65}\). Many ISCU Myopathy patients experience periods of muscle weakness, pain, and swelling associated with rhabdomyolysis and myoglobinuria, followed by muscle regeneration and resolution of these symptoms \(^{59,66}\). Previous skeletal muscle biopsy analyses revealed a distinctive pattern of biochemical and histological hallmarks in ISCU Myopathy, including deficiency of several mitochondrial iron sulfur (Fe-S) proteins as well as mitochondrial iron overload in affected myofibers \(^{15,63,66,67}\). The most striking deficiencies in Fe-S proteins were observed in aconitase and mitochondrial complex II/succinate dehydrogenase (SDH) \(^{58,63,67,68}\), though modest deficiencies in mitochondrial complex I, III, and the Rieske protein were also reported \(^{58,67}\).

The human ISCU protein works in a complex with the cysteine desulfurase NFS1, and the accessory proteins ISD11 and frataxin to assemble [2Fe-2S] and [4Fe-4S] clusters \(^{69,70}\), which are subsequently transferred to recipient apo-proteins aided by a chaperone complex that includes HSC20 and HSPA9 or a related HSP70 protein \(^{71}\). ISCU Myopathy is caused by a homozygous point mutation (g.7044 G>C) in intron 4 of the ISCU gene that results in retention of a 100bp fragment of intron sequence between exons 4 and 5 in the ISCU mRNA and inclusion of a premature translational stop codon in the aberrant open reading frame \(^{15,16}\). More recently, Kollberg et al. reported a more severe progressive myopathy associated with hypertrophic
cardiomyopathy, caused by the g.7044 G>C mutation on one allele in combination with a newly characterized heterozygous missense allele (c.149G>A) in ISCU exon 3.

Several recent studies have attempted to identify molecular features that may contribute to the unique tissue specificity and clinical phenotype of ISCU Myopathy. Sanaker et al. reported that the ratio between normal and mis-spliced ISCU mRNA species in patient muscle biopsies was different than that of patient myoblasts, fibroblasts and blood samples, suggesting that there were tissue-specific differences in RNA processing. Nordin et al. have built on this finding by showing that skeletal muscle ISCU protein levels were low in patient autopsy material as compared to heart, liver and kidney, while in another study they reported the identity of several muscle-specific cellular RNA-binding factors that influence the splicing of the mutant ISCU mRNA. Importantly, Kollberg et al., reported that an ISCU Myopathy patient biopsy of regenerating muscle showing much higher ISCU protein and SDH activity levels than a biopsy of mature muscle fibers taken nine years later after a rhabdomyolysis episode, suggesting that higher levels of functional ISCU protein are generated in newly-forming skeletal muscle.

Many questions remain regarding the tissue specificity and mechanism of ISCU Myopathy. What is the relationship between aberrant ISCU mRNA transcript expression and ISCU protein production in patient tissues? Are there additional factors in skeletal muscle that pre-dispose this tissue to the pathological effects of ISCU depletion? To address these and other questions, we evaluated ISCU mRNA and protein expression in four distinct cell types derived from ISCU Myopathy patients. We discovered surprisingly low ISCU and NFS1 protein levels in normal skeletal muscle as compared to other cell types. Aberrant ISCU mRNA and a truncated patient-specific ISCU protein were abundant in all cell types tested, but patient skeletal muscle
showed the lowest amount of normal ISCU mRNA and protein. Heterologous expression of the muscle-specific transcription factor MyoD1 in patient myoblasts reduced expression of normal ISCU mRNA, suggesting a mechanism by which undifferentiated muscle precursors might be temporarily spared from the pathology seen in mature, fully-differentiated muscle tissue. We also found that the Fe-S-dependent aconitases and IRP1/2 binding activities in patient primary cells were slow to recover from treatments with pro-oxidants, which caused a rapid and unexpected decrease in cellular ISCU abundance that could be prevented by antioxidant pre-treatment.
Materials and Methods

Patients, Tissue Biopsies, Primary Cultures

Three ISCU Myopathy patients were included in this study, aged 42, 42, and 66 years, as well as one 43 year old unaffected heterozygous offspring. Controls consisted of five healthy individuals, ages 22-47 years. Five patients of ages 29-63 years affected by un-related mitochondrial myopathy due to heteroplasmic mitochondrial DNA mutations were also included in some analyses. Skeletal muscle biopsies from vastus lateralis were obtained and prepared as described previously. Written informed consent was given by all individuals involved in the study in accordance with the Institutional Review Board of the University of Texas Southwestern Medical Center and the Presbyterian Hospital of Dallas. All primary cells were maintained in a low oxygen (5% O₂) humidified atmosphere at 37°C and 5% CO₂. Primary myoblast cell lines, a generous gift from Dr. Eric Shoubridge, were cultured as described previously, except that Lonza SKBM-2 culture medium was used. Primary fibroblasts were cultured in DMEM medium containing 5mM glucose and 110mg/L sodium pyruvate and supplemented with 10% FBS, and lymphoblasts were cultured in RPMI 1640 buffered with 25mM HEPES and supplemented with 10% FBS. For pro-oxidant exposures with H2O2 and DEA/NO (Sigma), the compounds were dissolved in minimal essential medium (Invitrogen) immediately before the start of the experiment, and added to cell cultures following two rinses with phosphate-buffered saline.

The lifespan of primary myoblast and fibroblast cultures was extended by infection with retrovirus LXSN16E6E7 which encodes the human papillomavirus E6 and E7 genes and the neomycin resistance cassette. Culture medium supernatants containing retrovirus were obtained from PA317 LXSN 16E6E7 cells (ATCC), sterile filtered, and transferred directly to
control and patient primary cultures daily for three days. All neomycin resistant cells were harvested and pooled after 6 days of subsequent incubation with 100µg/mL G418.

Animals

All procedures using C57BL/6 mice were approved by the NICHD Animal Care and Use Committee. Six month-old animals were deeply anesthetized by injection of an isotonic pentobarbital solution containing sodium heparin, and tissues were exsanguinated by cardiac perfusion with phosphate-buffered saline (PBS), harvested, and frozen in liquid N$_2$ immediately.

RNA Preparation, Northern Blots and qRT-PCR Analysis

RNA from biopsies and cell pellets was prepared using the mirVana™ RNA Isolation Kit (Ambion) according to the manufacturer’s instructions. qRT-PCR was performed using SYBR® Green (Applied Biosystems) according to the manufacturer’s instructions, following reverse transcription of total RNA into cDNA (Applied Biosystems). qRT-PCR primer sequences are listed in supplementary table 1. Relative transcript abundance was calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH as the internal control. Correct qRT-PCR product size was verified by agarose gel electrophoresis, and automated melting curves were generated and assessed after every experiment. Northern blots were performed with 1% agarose-formaldehyde gels as reported previously. DNA probes were produced by random priming of template sequences generated by RT-PCR and the Klenow DNA polymerase fragment (GE Healthcare Megaprime Kit), incorporating $\alpha$-$^{32}$P-deoxycytosine triphosphate (6000Ci/mmol; Perkin Elmer). Antisense RNA probes against ISCU mRNA sequences were synthesized using the MAXIScript T7 in vitro transcription kit (Ambion) incorporating $\alpha$-$^{32}$P-cytosine triphosphate (3000Ci/mmol; Perkin-
Elmer). The probe templates with T7 promoters were generated by PCR using an ISCU patient cDNA library or plasmids encoding the ISCU open reading frame. Primers used to make these probes are listed in Table 1.

Western Blots, Metabolic Labeling and Immunoprecipitation

SDS-PAGE and western blotting were performed as described previously using 1.5mm 4-12% pre-cast bis-Tris gels (Invitrogen). Mouse anti-MyoD1 and anti-SOD1 were from Abcam. Rabbit anti-citrate synthase was from Sigma-Aldrich. Mouse anti-SDH-B was from Mitosciences. Rabbit polyclonal antisera against ISCU, ISCS, IRP1, IRP2, and m-aconitase were produced from synthetic peptides as described previously. Rabbit polyclonal anti-ferrochelatase serum was a kind gift from Dr. Harry Dailey. Metabolic labeling and immunoprecipitation were carried out essentially as described.

Gene Expression Constructs

The human MyoD1 ORF in vector pSPORT6 was purchased from Invitrogen, amplified in E. coli, digested with EcoRI and XhoI, and sub-cloned into the pEBTetD mammalian episomal, tetracycline inducible expression vector. ISCU1 and ISCU2 ORF sequences were myc-tagged and amplified using PCR and reported ISCU ORF plasmid templates, restriction digested, and ligated into the pCEP4 episomal mammalian expression vector. Primary myoblast cell lines were transfected using the Neon™ Transfection System (Invitrogen), with settings of 1400 V, 20 ms pulse width, and 2 pulses per transfection. On the following day cells were permanently maintained in culture medium containing either 3µg/mL puromycin (pEBTetD) or 150µg/mL puromycin (pEBTetD) or 150µg/mL puromycin.
hygromycin (pCEP4). We found that heterologous gene expression from both plasmids in our primary cells was greatly enhanced by inclusion of 3 mM sodium butyrate in the culture medium.

**Immunofluorescence Microscopy**

Double immunofluorescent stainings of primary human myoblasts transfected with two different ISCU expression clones were performed following a general protocol \(^{82}\) with some modifications. Briefly, equal numbers of cells were attached onto pre-sterilized 12 mm coverslips. After washing, cells were fixed for 30 min with 4\% paraformaldehyde in PBS at room temperature. Cells were washed and incubated with 0.4\% Triton X-100, 4\% normal goat serum, and 2\% ovalbumin in PBS for blocking non-specific binding and cell membrane permeabilization for 30 min. Then cells were treated with ‘Endogenous biotin blocking kit’ (Invitrogen) following manufacturer’s protocol. Mouse anti-myc antibody (1/1000, Covance) mixed with either rabbit anti-SOD1 (1/300, RDI) or rabbit anti-Tom20 (1/300, Santa Cruz) was applied to cells and incubated for 30 min. After washing, cells were incubated with mixture of secondary antibodies (1/400, biotin-conjugated goat anti-mouse / Alexa fluoro 488-conjugated goat anti-rabbit) for 30 min. Anti-myc signal was detected using Cy3-streptavidin. Finally, cells were mounted with anti-fading media containing DAPI (Vector Laboratories) and analyzed for ISCU expression in different subcellular compartments using a confocal microscope (Olympus Fluoview).

**Aconitase In-Gel Assay and Electrophoretic Mobility Shift Assay (EMSA)**
Aconitase was assayed using a coupled assay following native PAGE separation, as described previously. IRP-IRE binding activity was determined by EMSA using a $^{32}$P-labeled ferritin IRE probe, as described previously.
Results

Low ISCU and NFS1 protein levels in skeletal muscle tissue

In order to gain insight into possible explanations for the tissue specificity of ISCU Myopathy, we first set out to examine ISCU protein expression in various control and patient cell types. Biopsies from vastus lateralis of either an ISCU Myopathy patient or a healthy control showed markedly lower ISCU protein levels in comparison to cultured human myoblasts, fibroblasts and lymphoblasts (Figure 1A, S1). The ISCU binding partner NFS1, which provides inorganic sulfide for formation of Fe-S clusters on ISCU, was also relatively low in the control biopsy, and was undetectable in the patient biopsy (Figure 1A). Notably, NFS1 was mildly decreased in patient myoblasts and fibroblasts as compared to controls as well (Figure 1A). We also checked expression of the recipient Fe-S-containing enzymes aconitase and succinate dehydrogenase subunit B (SDH-B), which rely on ISCU and NFS1 for synthesis and donation of Fe-S clusters. Mitochondrial aconitase and SDH-B proteins were abundantly expressed in the control muscle biopsy, while SDH-B was decreased to undetectable levels in the ISCU Myopathy patient biopsy (Figure 1B). ISCU mRNA in the control muscle biopsy was not low relative to the other cell types (Figure 1C). In tissues taken from a healthy control mouse, ISCU and NFS1 protein levels were also lowest in skeletal muscle, whereas ISCU levels were highest in heart (Figure 1D) and were much higher in the liver, kidney and brain. Again, SDH-B and mitochondrial aconitase were abundant in both mouse heart and skeletal muscle (Figure 1D). Together, these data demonstrate that critical components of the mitochondrial Fe-S assembly
machinery are markedly low in a tissue (skeletal muscle) in which their need is undoubtedly great.

**Altered ISCU mRNA Expression in ISCU Myopathy Patient Muscle Biopsies**

In order to evaluate ISCU mRNA expression in control and ISCU Myopathy patient samples, we developed DNA and RNA probes complementary to several regions of the ISCU mRNA transcript, including sequences covering exon 2-4 or exon 5, and the region encompassing the patient-specific exon 4A and several hundred base-pairs of 3’sequence contained in intron 4 (Figure 2A). Northern blots demonstrated the presence of one predominant mRNA species in control RNA extracts (transcript I), and two additional patient-specific transcripts in ISCU Myopathy samples, denoted as transcripts II and III (Figure 2B, S2A,B). RT-PCR and sequencing of three distinct isolated clones representing these bands confirmed that transcript I represented the correctly-spliced ISCU mRNA transcript, while transcript II corresponded to an mRNA species in which the 100bp previously denoted as exon 4A was included, and transcript III contained exon 4A as well as the remaining 1073bp intronic sequence that was 3’ of exon 4A (Figure 2C). Quantitative RT-PCR further confirmed high expression of exon 4A as well as downstream regions of intron 4 in patient muscle biopsies, (Figure 2D, S3). Notably, the total amount of ISCU mRNA containing exon 5 was reduced to about 50% of control levels (Figure 2D). Using high affinity ³²P-labeled RNA probes, we observed several additional high molecular weight patient-specific mRNA species in fibroblasts, likely indicating the existence of partially-spliced ISCU mRNA intermediates that were not observed in the patient muscle biopsies (Figure 2E,F). Finally, we compared the pattern of ISCU mRNA
expression in patient muscle biopsies directly to that of cultured patient-derived myotubes and fibroblasts, and found that the residual amount of normal-sized ISCU mRNA (transcript I) was clearly lowest in the patient muscle biopsy (Figure 2G). Further northern blot analysis using an RNA probe directed against ISCU exon 2-4 demonstrated that total ISCU mRNA expression was decreased in all three patient cell types relative to respective controls, as well as strong expression of the ISCU Myopathy-specific ISCU mRNA transcripts II and III (Figure S2C-G).

**Decreased expression of full-length ISCU protein leads to low ISCU levels in cultured ISCU patient primary cells**

To investigate the relationship between altered ISCU mRNA expression and aberrant ISCU protein expression in ISCU Myopathy patient tissues, we conducted metabolic labeling experiments with cultured myoblast, fibroblast, and lymphoblast cells derived from patients and controls. Newly-synthesized ISCU protein was extracted and immunoprecipitated from radiolabeled cells, revealing a band of ~13kDa that corresponded with the predicted size of the truncated patient-specific ISCU protein following import into the mitochondrion and cleavage of the mitochondrial transit peptide (Figure 3A-C). We also found decreased amounts of the full-sized ~15kDa mitochondrial ISCU band in the myoblasts and fibroblasts, relative to respective controls (Figure 3A-C). A pulse-chase experiment in patient myoblasts demonstrated that the truncated ISCU protein had an extremely short half-life (<30min), while the full-sized mitochondrial ISCU was relatively stable throughout the two hour experiment (Figure 3D). Finally, western blotting demonstrated that total ISCU protein levels were decreased substantially in myoblasts and fibroblasts and minimally in lymphoblasts relative to controls.
(Figure 3E, Figure S4). These data demonstrated that aberrantly spliced ISCU mRNA gave rise to synthesis of the unstable truncated ISCU protein in all patient cell types that were tested, while residual levels of the normal mitochondrial ISCU protein varied substantially between cell types.

**Mitochondrial ISCU restores Fe-S protein activities and iron homeostasis in ISCU myopathy patient myoblasts**

The ISCU protein is found in two distinct isoforms in human cells, namely a low abundance cytosolic isoform known as ISCU1, and a far more abundant mitochondrial isoform known as ISCU2. We stably expressed ISCU1 and ISCU2 in patient-derived myoblast cell lines to test whether these proteins could rescue defects in Fe-S cluster proteins and cellular iron metabolism in these cells. Using confocal microscopy, we verified that the cytosolic and mitochondrial forms of our ISCU1 and ISCU2 constructs localized as predicted (Figure 4A,B). Mitochondrial and cytosolic aconitase activities were enhanced by expression of mitochondrial ISCU in patient cells, whereas IRE binding activity of IRP1 decreased (Figure 4C). Furthermore, IRP2 protein levels decreased in ISCU2-expressing cells, and more subtly in ISCU1-expressing cells, while H-ferritin protein levels increased upon expression of ISCU2, consistent with correction of cytosolic iron depletion that frequently occurs in conjunction with disturbed mitochondrial FeS synthesis. Together, these data demonstrate that mitochondrial and cytosolic Fe-S enzymes and iron homeostasis in patient ISCU Myopathy patient myoblasts can be restored by increasing mitochondrial ISCU levels.
MyoD-Induced Muscle Lineage Differentiation Causes Decreased Levels of Normally-Spliced ISCU mRNA in Patient Myoblasts

The data here and in other studies \(^{72,73}\) suggest that greater amounts of normally spliced ISCU mRNA exist in ISCU Myopathy patient tissues such as heart, liver, fibroblasts or myoblasts as compared to skeletal muscle. In order to further explore this tissue specificity, we generated stably transfected patient myoblasts expressing the muscle-specific MyoD1 transcription factor driven by a tetracycline-inducible promoter. In these cells, doxycycline and sodium butyrate treatment resulted in a ~50-fold induction of MyoD1 mRNA expression, and a concomitant ~6-fold induction of myosin heavy chain 1 (MYH1) mRNA relative to un-induced cells, indicating initiation of the muscle differentiation program (Figure 5A). Western blots confirmed strong induction of MyoD1 protein expression in transfected control and patient cell lines (Figure 5B). RT-PCR of ISCU mRNA using primers flanking the ISCU exon 4-5 boundary demonstrated a shift in the relative abundance of normal vs. abnormally-spliced ISCU mRNA in ISCU Myopathy patient cells (Figure 5C). The amount of normally-spliced ISCU mRNA (transcript I) decreased, whereas the amount of patient-specific ISCU mRNA containing the 100bp exon 4A (transcript II) increased with MyoD induction (Figure 5C). We followed ISCU mRNA expression during induction of MyoD1 in these cells for 0, 12, 24, or 48 hours, and we observed a relative increase in expression of transcript II and an eventual decrease in transcript I by northern blot (Figure 5D) and RT-PCR (Figure 5E).

Impaired synthesis of ISCU protein renders patient myoblasts and fibroblasts more sensitive to oxidative stress
Skeletal muscle has long been known to be the source of significant reactive oxygen species (ROS) during both rest and exercise. This sensitivity to ROS led us to ask whether ISCU protein deficiency in ISCU Myopathy patient primary cells might render them more sensitive to oxidative stress. To explore this hypothesis, we exposed control and patient myoblasts to a 1 hour hydrogen peroxide pulse, followed by incubation/recovery in normal medium for four or eight hours. Results of this experiment showed that the recovery of mitochondrial and cytosolic aconitases was impaired in the patient myoblasts as compared to control cells (Figure 6A). IRE binding activity in patient and control cells was rapidly activated by H2O2 treatment, and the activation was greater and more sustained in the patient cells (Figure 6A). Unexpectedly, ISCU protein levels in both control and patient cells were significantly diminished by H2O2 treatment, with ISCU levels in the patient cells falling to nearly undetectable levels following H2O2 exposure (Figure 6A). We also explored the effect of nitric oxide (NO) on control and patient myoblasts by treatment with the NO donor DEA/NO for one hour, followed by a four and eight hours of recovery. ISCU protein levels were not measurably decreased by DEA/NO, but recovery of cytosolic and mitochondrial aconitases was again impaired in the patient cells, and greater and more sustained activation of IRE binding activity was observed as well (Figure 6B). Since ISCU protein in patient cells appeared to be readily decreased by H₂O₂, we tested the ability of antioxidants to prevent this effect. Patient myoblasts were pre-treated with either 0.2 or 0.5mM Na ascorbate, or with 0.5mM reduced glutathione for 12 hours, washed twice with normal medium, and exposed to 1mM H₂O₂ for 1 hour. Immediately following H₂O₂ exposure, the cells were harvested and ISCU protein levels were
measured by western blot (Figure 6C). Our data showed that ascorbate, but not glutathione, prevented the rapid H2O2-mediated decrease in ISCU protein levels.

Mammalian aconitase is known to be highly sensitive to an enriched O2 atmosphere. We tested the effect of a 16 hour exposure to 95% O2 on aconitase activities, IRE binding activities, and ISCU protein levels in patient and control fibroblasts. Surprisingly, ISCU protein levels in patient cells were reduced by high oxygen exposure, and remained low for the duration of the experiment (Figure 6D). Mitochondrial aconitase activity in patient cells also diminished and remained low for the duration of the experiment (Figure 6D). To further test the influence of mitochondrial and cytosolic ISCU expression on Fe-S proteins and IRE binding activity in patient myoblasts, cells were exposed to 95% O2 for 16 hours, followed by recovery in 5% O2 for 24 hours. After this treatment, patient cells expressing mitochondrial ISCU showed significant recovery of aconitase activities and lower IRE binding activities (Figure 6E), whereas mitochondrial and cytosolic aconitase activities remained low (Figure 6E) in cells expressing cytosolic ISCU, indicating that the cytosolic ISCU isoform cannot alone rescue the mitochondrial FeS synthesis and cytosolic iron homeostasis problems of ISCU deficient cells.
Discussion

Since the identification of the single intronic point mutation in the ISCU gene that underlies the pathophysiology of ISCU Myopathy, several groups have suggested that tissue-specific splicing explains the muscle-specific disease phenotype. In this study, we have extended these findings and discovered further potential reasons for tissue specificity. We found that ISCU and NFS1 protein levels were surprisingly low in human and mouse skeletal muscle in comparison to other tissues. Unexpectedly, northern blots demonstrated that aberrant ISCU mRNA transcripts were generated in all tested patient cell types, including fibroblasts and lymphoblasts, which do not manifest a phenotype in patients. However, expression of the normal ISCU mRNA was clearly lowest in patient skeletal muscle, even though ISCU protein levels were clearly depleted in several different patient cell types, and all patient cells abundantly expressed a truncated and unstable patient-specific ISCU protein. The ISCU mRNA expression pattern in patient myoblasts more closely resembled that of patient skeletal muscle biopsies following over-expression of the muscle-specific transcription factor MyoD1. Finally, biochemical analysis of Fe-S enzymes in primary myoblasts and fibroblasts revealed that patient cells were slow to recover from oxidative stress challenges, a defect that could be rescued by expression of mitochondrial ISCU. Surprisingly, cellular levels of ISCU protein were rapidly depleted during these exposures, suggesting that sensitivity of ISCU protein to oxidative stress might further diminish precariously low levels of normal ISCU in patient skeletal muscles.

We were surprised to find such low levels of the core Fe-S assembly machinery components ISCU and NFS1 in human and mouse skeletal muscle samples, as skeletal muscle is a dynamic organ system which experiences rapid and dramatic changes in energy metabolism.
and oxygen consumption during exercise and rest. Muscles also possess the ability to utilize several different metabolic pathways to produce ATP depending on the myofiber type and level of contractile activity, ranging from almost exclusive dependence on fatty acid oxidation during times of rest to predominant utilization of glucose oxidation pathways during periods of maximal exertion.\textsuperscript{89} Cellular respiration requires many different Fe-S-containing proteins, and therefore the need for Fe-S cluster synthesis and repair is likely to be great in this tissue. In line with this, we found abundant expression of mitochondrial aconitase and SDH-B in control human and mouse skeletal muscle samples, despite puzzlingly low ISCU and NFS1 levels. Although we do not know why ISCU and NFS1 are expressed at low levels in skeletal muscle, these inherently low levels of Fe-S cluster assembly machinery in normal skeletal muscle likely pre-dispose skeletal muscle to the effects of further ISCU depletion.

Abnormal splicing also clearly contributes to the phenotype of patients, although our results indicate that there is a continuum in the spectrum of abnormal splicing, and we found evidence for abnormal splicing in two other patient cell types. Previous RT-PCR-based studies by two groups have reported that ISCU Myopathy patient skeletal muscle shows a high proportion of ISCU mRNA containing the 100bp patient-specific exon 4A.\textsuperscript{72,73} In our study, northern blots and qRT-PCR revealed that all tested patient cells abundantly express an ISCU mRNA containing the entire intronic sequence downstream of the patient-specific exon 4A (‘Transcript III’; see Figure 1C). This longer mRNA transcript might be significantly more stable than the canonical patient-specific transcript (Transcript II), since the 56bp of sequence between the premature stop codon and the exon 5 junction make this molecule a good candidate for nonsense-mediated decay (NMD).\textsuperscript{90}
Regardless of their cellular fate, both patient-specific ISCU mRNAs encode the same truncated ISCU protein, which we found to be very short-lived and detectable only by metabolic labeling. Given the instability of this truncated ISCU protein, the patient cells must rely on the decreased residual amounts of normal ISCU mRNA transcript (Transcript I) to produce functional ISCU protein. Accordingly, patient myoblasts and fibroblasts showed decreased synthesis of stable full-length ISCU protein. However, the striking distinction between patient primary cells and patient muscle biopsies was in the vanishingly small amount of normal ISCU mRNA that remained in the biopsies. To explore this difference, we expressed MyoD1 in patient-derived cells to induce myogenic differentiation. This strategy has been used previously in several cell types to induce gene expression more closely resembling that of mature myofibers. Our observation that the proportion of normally-spliced ISCU mRNA levels decreased in patient cells following MyoD expression is likely a result of altered expression of one or more RNA binding proteins and splicing factors. Such factors may include IGF2BP1, RBM39, and PTBP1, which were recently identified by Nordin et al. to influence the retention of exon 4A during splicing of the mutant ISCU mRNA.

Although clearly lower in patient vs. control myoblasts and fibroblasts, ISCU protein levels in all tested cell lines was far higher than in control and patient skeletal muscle biopsies. This finding may offer insight into the non-progressive nature of the ISCU Myopathy disease, since myofiber regeneration processes in the patients would allow for fusion and differentiation of new myoblasts, and we have found here that myoblasts possess significantly higher initial ISCU protein levels as compared to mature patient skeletal muscle. The higher ISCU expression in myoblasts would allow regenerating patient muscles a window of time in which ISCU
expression was sufficiently high to allow for more normalized levels of Fe-S protein expression. This idea is in line with the recent report of Kollberg et al. (2010), which illustrated significantly higher levels of ISCU protein expression and succinate dehydrogenase activity in the regenerating muscle of an ISCU myopathy patient who was recovering from an acute attack of rhabdomyolysis and myoglobinuria. A biopsy taken from this same patient 9 years later showed significantly lower ISCU protein levels and SDH activity, along with very few regenerating fibers.

Previous work has shown that the mitochondrial Fe-S cluster assembly machinery is essential for maintenance of iron homeostasis in both the mitochondrial and cytosolic cellular compartments. siRNA-mediated depletion of ISCU in the mitochondria of human tissue culture cells resulted not only in decreased mitochondrial aconitase activity and mitochondrial iron overload, but also in cytosolic iron depletion as demonstrated by decreased cytosolic aconitase activity and activation of IRP1/2. In contrast, depletion of the cytosolic isoform of ISCU resulted in impaired assembly/repair of cytosolic aconitase, whereas mitochondrial aconitase was unaffected. We found that heterologous expression of the mitochondrial ISCU isoform caused increased m- and c-aconitase activities in ISCU Myopathy patient myoblasts. Patient cells expressing mitochondrial ISCU also behaved more like healthy control myoblasts in response to high O2 treatment, while cells expressing cytosolic ISCU showed no improvement in aconitase activity levels. Together, these data suggest that depletion of mitochondrial ISCU is the cause of the decreased mitochondrial and cytosolic aconitase activities and cytosolic iron deficiency in affected ISCU Myopathy patient cells.
Skeletal muscle is unique in that it is subjected to large transient bursts of reactive oxygen species during both low- and high-intensity exercise\textsuperscript{85,86,93,94} and even during prolonged inactivity\textsuperscript{87}. We report here that ISCU Myopathy patient cells showed impaired recovery of aconitase activities following exposure to three distinct pro-oxidants (H$_2$O$_2$, DEA/NO, and high O$_2$). Though artificial, these oxidative treatments might mimic the transient bursts of reactive oxygen species production that are known to occur in active skeletal muscle\textsuperscript{85,86,95}. A surprising result of these experiments was the rapid decrease in ISCU protein levels following H$_2$O$_2$ or high O$_2$ treatment. We hypothesize that under these conditions Fe-S clusters forming on ISCU undergo rapid oxidative modifications, as previous work has shown that H$_2$O$_2$ can readily oxidize [2Fe-2S] clusters on \textit{E. coli} IscU, resulting in release of iron and sulfide\textsuperscript{96}.

If human ISCU is more unstable when devoid of an Fe-S cluster, then oxidant-mediated cluster dissolution would lead to a decreased protein half-life, an effect seen in several other mammalian Fe-S proteins\textsuperscript{60,78}. Alternatively, ISCU degradation might be signaled by oxidant-induced modification of amino acid residues of human ISCU, such as disulfide bond formation or irreversible oxidation of active site cysteine thiols to sulfinic acid (S-O$_2$H). Jang and Imlay recently reported that the \textit{E. coli} Isc system is inactivated by low levels of H$_2$O$_2$, likely by disruption of Fe-S cluster assembly on IscU or prevention of cluster transfer from IscU\textsuperscript{97}. In this case, the \textit{suf} Fe-S assembly/repair operon was activated to restore Fe-S enzyme activities. As mammalian cells are not known to possess homologs of the \textit{suf} genes, we wonder whether other mechanisms exist in mammalian cells to synthesize and repair Fe-S clusters during periods of stress.
Our experiments suggest that the steady-state level of ISCU protein in various tissues could be influenced by the amount of ROS generated in a given tissue microenvironment. Under this hypothesis, ROS-mediated decreases in ISCU protein levels could be avoided by removal of the oxidative insult using antioxidants. Accordingly, the antioxidant sodium ascorbate prevented the H$_2$O$_2$-induced decrease in ISCU protein in our patient cells. We speculate that prevention of ISCU degradation might be an important component of the protective effects that antioxidants have on muscle ROS levels during and after exercise $^{94,98}$. This finding merits the consideration of whether targeted antioxidant therapy might be of benefit to ISCU Myopathy patients.

In summary, we suggest that the tissue specificity in ISCU myopathy patients is attributable to several factors, including tissue and differentiation-specific abnormal splicing, low natural protein abundance, and unusual sensitivity to oxidative stress, which is a pronounced consequence of skeletal muscle activity. These factors synergize to lead to significant and pathological deficiency of ISCU in mature muscle cells, whereas other cell types possessing reduced ISCU due to abnormal splicing are more robust because they express more ISCU protein and are not exposed to bursts of oxidative stress.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.
Figure 1: Steady-State ISCU and NFS1 protein levels are inherently low in muscle tissue. (A) Western blots were used to assess protein levels in lysates from control and patient vastus lateralis biopsies and primary cells. Short and long film exposures of ISCU and NFS1 western blots are shown for completeness. Citrate synthase serves as a loading control for mitochondrial protein. All proteins were probed on the same filter. (B) ISCU mRNA levels were assessed by northern blot in a healthy control vastus lateralis biopsy and in primary cells derived from a healthy individual. (C) Western blots assessed protein levels present in lysates derived from various mouse tissues.
Figure 2: Altered ISCU mRNA Expression Patterns in ISCU Myopathy Patients. (A) A schematic representation of ISCU mRNA showing the single intronic point mutation within the fourth intron, canonical and aberrant splicing patterns, and regions of northern blot probe complementarity. (B) Hybridization of ISCU-specific $^{32}$P-labeled DNA probes with control and
patient *vastus lateralis* biopsy RNA demonstrated normal (I) and additional patient-specific (II,II) ISCU mRNA bands. (C) RT-PCR and sequencing using primers flanking ISCU exons 4 and 5 confirmed the identity of the three major ISCU bands (I-III). (D) qRT-PCR analysis of three ISCU Myopathy patients and seven control biopsies using primer sets for ISCU exon 4A, downstream intron 4 sequences, and exon 5. (E,F) Northern blot analysis of ISCU mRNA expression in control and patient muscle biopsies and fibroblasts using high specific activity RNA probes revealed several larger ISCU-specific mRNA bands in ISCU Myopathy patient fibroblasts. Methylene blue-stained 18S ribosomal RNA serves as a loading control. (G) Northern blot analysis of control and patient muscle biopsies alongside myotubes and fibroblasts demonstrate a decreased residual level of normally-spliced ISCU mRNA in the patient muscle biopsy.
Figure 3: An unstable truncated patient-specific ISCU protein is produced in ISCU Myopathy patient myoblasts, fibroblasts and lymphoblasts, and total ISCU protein is depleted to different degrees. Metabolic labeling of control (C1,C2) and patient (P1,P2) primary cells with $^{35}$S-cys and $^{35}$S-met for 30 min followed by immunoprecipitation and SDS-PAGE revealed the presence of a truncated mitochondrial ISCU band as well as decreased synthesis of the normal-sized mitochondrial ISCU in patient myoblasts (A), fibroblasts (B), and lymphoblasts (C). (D) A pulse-chase experiment was performed to follow the fate of the normal-sized and truncated ISCU protein products over time (see Materials and Methods). (E) Western blots using an antibody against ISCU demonstrated decreased total ISCU protein levels in patient primary cells.
Figure 4: Expression of mitochondrial ISCU restores Fe-S protein activities and iron homeostasis in ISCU Myopathy patient-derived myoblasts. (A,B) Primary patient myoblasts were stably transfected with a plasmid encoding myc-tagged cytosolic ISCU (ISCU1) or mitochondrial ISCU (ISCU2). Proper sub-cellular localization was verified by double-immunostaining with SOD1 (cytosol) and TOM20 (mitochondrion), and nuclei were stained with DAPI. © ISCU, IRP2, and ferritin protein levels were assessed by western blot, and mitochondrial and cytosolic aconitase activity levels and IRP-IRE binding activity were assessed as described in Materials and Methods.
Figure 5: MyoD-Induced Differentiation in Patient Myoblasts Leads to Decreased Residual Levels of the Normally-Spliced ISCU mRNA Isoform. (A) Pooled clones of control and patient primary myoblasts stably harboring the episomal plasmid pEBTetD-MyoD (see Materials and Methods) were incubated in growth medium or differentiation medium in the presence or absence of 1µg/mL doxycycline (DOX) and 3mM sodium butyrate for 48 hours. MyoD1 and MYH1 mRNA expression was measured by qRT-PCR. Two-tailed t-tests were used to evaluate statistical significance (**P<0.01) (B) Inducible MyoD protein expression in control and patient myoblasts was verified by western blot. (C) The pattern of ISCU mRNA expression in patient myoblasts in the presence or absence of MyoD over-expression was assessed by qualitative RT-PCR using primers flanking ISCU exons 4 and 5. The control is undifferentiated myoblast mRNA from a healthy individual. PCR bands I-III correspond to the bands identified in
Figure 1C. (D) The time-course of ISCU mRNA expression in differentiating myoblasts was followed by northern blot using a $^{32}$P-labeled ISCU RNA probe. (E) RT-PCR using primers flanking ISCU exons 4 and 5 was performed on the same RNA samples as in panel D.
Figure 6: Impaired Synthesis of ISCU Protein Renders Patient Myoblasts and Fibroblasts More Sensitive To Oxidative Stress. (A) Control and patient myoblasts were pulsed with H$_2$O$_2$ for 1 hour, followed by a chase in normal culture medium for four or eight hours. ISCU protein levels, mitochondrial and cytosolic aconitase activity levels, and IRP-IRE binding activity were measured. (B) Control and patient myoblasts were also tested for their ability to recover from a one hour pulse with 1mM diethylamine-nitric oxide (DEA/NO). (C) Pre-incubation of primary patient myoblasts with sodium ascorbate (vitamin C) prevented H$_2$O$_2$-mediated depletion of ISCU protein. Pre-incubation with reduced glutathione (GSH) had no effect on ISCU depletion.
(D) Control and patient primary fibroblasts were tested for their ability to recover aconitase activity and IRP-IRE binding activity in a 5% O$_2$ atmosphere, following a 16 hour challenge in a 95% O$_2$ / 5% CO$_2$ atmosphere.  (E) Control and patient myoblasts expressing either mitochondrial or cytosolic ISCU were grown in a 95% O$_2$ atmosphere for 16 hours, followed by recovery at 6% O$_2$ for 24 hours.
Figure 7: The impact of aberrant ISCU mRNA expression on ISCU protein synthesis, and the cellular response to oxidative stress. (A) An intronic point mutation (g.7044 G>C) leads to decreased expression of the normally-spliced ISCU mRNA, and the appearance of two predominant patient-specific ISCU mRNA transcripts in all tested patient tissues, both of which encode a truncated ISCU protein that is rapidly degraded after synthesis. MyoD expression and maturation of skeletal muscle myofibers leads to further decreases in the normally-spliced ISCU mRNA, eventually leading to pathologically low ISCU protein levels in mature skeletal muscles. (B) Generation of reactive oxygen species such as hydrogen peroxide and superoxide can exacerbate Fe-S cluster deficiency in patient skeletal muscles by causing further depletion of ISCU protein, which cannot be rapidly re-synthesized due to the ISCU mRNA splicing defect.
Figure S1: ISCU protein expression in skeletal muscle biopsies and myoblasts. ISCU protein levels in skeletal muscle and in primary myoblasts were compared directly by western blot. Sample M1 was from a mitochondrial myopathy patient with a heteroplasmic mtDNA deletion, C1-C3 were from healthy control individuals, H1 was an individual heterozygous for the ISCU Myopathy mutation, and P1-3 were from ISCU Myopathy patients. Primary myoblast samples were from proliferating myoblast cultures (CtlA, PatA), or from myoblast cultures incubated in low serum conditions for five days (CtlB, PatB). A catalase immunoblot from the same nitrocellulose filter serves as a loading control.
Figure S2: ISCU mRNA expression in myoblasts, fibroblasts, and lymphoblasts derived from ISCU Myopathy patients and a heterozygous individual. Hybridization of ISCU-specific $^{32}$P-labeled DNA and RNA probes with control, heterozygote, and patient RNA extracts.
(A,B) Northern blot of *vastus lateralis* biopsy RNA using DNA probes demonstrated normal (I) and additional patient-specific (II,II) ISCU mRNA bands. (C) comparison of ISCU mRNA expression in various tissues using a $^{32}$P-labeled RNA probe. (D,E) ISCU mRNA expression in fibroblast RNA samples. (F,G) ISCU mRNA expression in myoblast samples subjected to either five or 21 days of low-serum conditions.
Figure S3: qRT-PCR evaluating expression of six different regions of the ISCU mRNA transcript in muscle biopsies. qRT-PCR analysis of three ISCU Myopathy patients and seven control biopsies using primer sets for ISCU introns 2 and 3, and four different regions of intron 4. Ct values were normalized to Ct values obtained using a primer set covering ISCU exons 2-3.
Figure S4: ISCU protein expression in myoblasts, fibroblasts, and lymphoblasts derived from ISCU Myopathy patients and a heterozygous individual. ISCU protein levels were assessed by western blot in several different ISCU Myopathy patient primary cell lines, a heterozygous cell line, and cell lines derived from healthy control individuals. (A) Undifferentiated myoblasts, (B) fibroblasts, (C) immortalized B lymphoblasts. Nitrocellulose filters were re-probed with either SOD2, tubulin, or citrate synthase to assess equal loading.
### Table 1

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Table 1: PCR Primer sequences utilized in this study.
CHAPTER 3

Aberrant iron homeostasis, oxidative fiber enrichment, and activation of ketogenesis in muscle tissue of ISCU Myopathy patients

Short Title: Molecular Pathophysiology of ISCU Myopathy

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Abstract

ISCU Myopathy, a disease characterized by life-long exercise intolerance and impaired mitochondrial oxidative metabolism, is caused by deficiency of the Fe-S cluster scaffold protein ISCU. We performed gene expression analysis on muscle biopsies from ISCU Myopathy patients to gain insight into the molecular processes that are perturbed due to impaired Fe-S cluster assembly in human muscle. We found that ISCU depletion led to increased expression of the mitochondrial iron importer MFRN2 and the rate-limiting heme biosynthetic enzyme ALAS1. Gene expression and histochemical analyses demonstrated a shift in patient muscle composition characterized by fewer glycolytic muscle fibers, more oxidative fibers, and increased muscle capillary perfusion. Paradoxically, mitochondrial fatty acid uptake and oxidation genes were coordinately up-regulated in patient muscles despite dramatic impairments of mitochondrial aconitase and succinate dehydrogenase activities. The ketogenic enzymes HMGCS2 and BDH1 were also significantly up-regulated, as was the secreted starvation response hormone, FGF-21. We propose that enhanced fatty acid oxidation in the setting of impaired TCA cycle flux results in over-accumulation of acetyl-coenzyme A in affected patient muscle fibers. In response, ketogenesis is initiated in order to restore free coenzyme A levels and shunt the products of fatty acid oxidation to distal respiration-competent tissues in the body.
Introduction

“Myopathy with deficiency of succinate dehydrogenase and aconitase”, also known as ISCU Myopathy, is a rare autosomal recessive hereditary disease found mainly in individuals of northern Swedish descent, and is characterized by life-long exercise intolerance\textsuperscript{59,64}. Trivial bouts of exercise in these patients cause dramatically increased heart rate and palpitations, dyspnea, muscle fatigue, and lactic acidosis\textsuperscript{59,65}. Muscle hypertrophy has also been observed in many ISCU patients, particularly in the calf muscles\textsuperscript{59,63,67}. Many ISCU Myopathy patients have also experienced sporadic activity-related periods of severe muscle weakness and pain, associated with rhabdomyolysis and myoglobinuria, eventually followed by muscle regeneration and resolution of these symptoms\textsuperscript{59,66}. Two groups reported the genetic cause of ISCU Myopathy, mapping the lesion to single intronic point mutation (g.7044 G>C) in the ISCU gene that dramatically affected the ISCU mRNA splicing pattern\textsuperscript{15,16}, resulting in retention of a 100bp fragment of intronic sequence and inclusion of a premature translational stop codon.

Analysis of muscle biopsies taken from ISCU Myopathy patients has revealed a unique pattern of biochemical and histological features, characterized by profound deficiency of several mitochondrial iron sulfur (Fe-S) proteins as well as mitochondrial iron overload\textsuperscript{15,63,66,67}. The most striking deficiencies in Fe-S proteins are observed in aconitase and mitochondrial complex II/succinate dehydrogenase (SDH), where normalized enzyme activity values were reported to be just 10-20\% of healthy controls\textsuperscript{58,63,67,68}, though milder deficiencies in mitochondrial complex I, III, and the Rieske protein were also reported\textsuperscript{58,67}. Notably, capillary endothelial cells in a patient muscle biopsy did not show depleted SDH activity, underscoring the strict tissue
specificity of the disease. Additionally, an ISCU patient muscle biopsy taken shortly after an episode of rhabdomyolysis showed regenerating muscle fibers with much higher SDH activity than mature muscles taken nine years later, suggesting that muscle precursor cells may also be spared from the disease.

Although the reason(s) for the restriction of the clinical phenotype of ISCU Myopathy to skeletal muscle remain unclear, several recent studies have shed light on molecular features of the disease that may contribute to the tissue specificity. Sanaker et al. (2010) reported that the ratio between normal and mis-spliced ISCU mRNA species in patient muscle biopsies is quite different than that of myoblasts, fibroblasts and blood samples, suggesting tissue-specific differences in RNA processing. Nordin et al. (2011) have built on this finding, showing low relative ISCU protein levels in patient skeletal muscle as compared to patient heart, liver and kidney, with tissue iron accumulation occurring only in skeletal muscle. Finally, Kollberg et al. (2009) reported two brothers possessing compound heterozygous ISCU mutations including one Swedish g.7044G>C allele as well as a new missense mutation allele (c.149G>A) in ISCU exon 3 that resulted in a more severe progressive myopathy with hypertrophic cardiomyopathy, thus extending the tissue involvement of Fe-S cluster deficiency to cardiac muscle.

Despite the recent advances in the pathophysiology of ISCU Myopathy, the events that lead to mitochondrial iron overload, the cellular responses to impaired energy production, and the molecular signals underlying the tissue remodeling and hypertrophy remain unclear. Here, we compared global gene expression patterns in ISCU patient muscle biopsies to healthy controls, and to patients with un-related mitochondrial myopathies. Our results suggest that prolonged ISCU deficiency causes a distinct expression pattern of iron-related genes, as well as a
broad-scale shift in muscle fiber type and fatty acid metabolism gene expression. We found strong evidence for muscle tissue remodeling characterized by fewer glycolytic muscle fibers and more oxidative fibers, accompanied by a marked increase in muscle capillary perfusion. These morphological alterations were accompanied by coordinated up-regulation of genes involved in mitochondrial fatty acid uptake and metabolism, despite the known impairment in TCA cycle function. Finally, key proteins normally involved in the starvation response and ketogenesis were dramatically up-regulated in the ISCU Myopathy patient muscle biopsies. This coordinated induction of fatty acid oxidation and starvation-induced genes in patient muscle fibers likely reflects a response to intracellular energy deficiency. Furthermore, activation of ketogenesis in affected patient muscle fibers may represent an effort to dispose of excess fatty acid-derived acetyl-CoA, to maintain adequate free coenzyme-A levels when the flux of carbon substrates through the TCA cycle is impaired.
**Results**

**Global gene expression analysis in ISCU Myopathy patient muscle biopsies**

We performed microarray analysis on ISCU Myopathy patient muscle biopsies in order to identify pathways involved in the cellular response to Fe-S cluster deficiency and impaired oxidative metabolism. Analysis of the microarray data using iProXpress, a tool for exploration of large-scale expression data, suggested that there were alterations in oxidative phosphorylation, glycolysis, fatty acid metabolism, and peroxisome proliferator-activated receptor (PPAR) signaling (Table 1). DAVID ontology analysis demonstrated that a large number of mitochondrial genes were up-regulated, while gene groups implicated in muscle contraction were down-regulated (Figure 1A), likely reflecting the changes in the proportion of glycolytic to oxidative muscle fibers discussed below. Finally, Ingenuity Pathway Analysis also indicated remodeling of glycolysis, oxidative phosphorylation, and fatty acid metabolism, the latter two of which were largely up-regulated (Figure 1B). Subsets of the genes representing these functional pathways were selected for analysis by qRT-PCR and other methods reported below.

**Up-regulation of key iron metabolism genes in ISCU-depleted biopsies and cells**

ISCU depletion results in deficiency of Fe-S protein activities, altered cellular iron homeostasis, and mitochondrial iron overload in cultured cells and in affected ISCU Myopathy patient skeletal muscle. Our microarray analysis suggested that expression of several iron homeostasis-related genes was altered in ISCU Myopathy patient muscle biopsies (Table S1), and several of these changes were verified with qRT-PCR (Figure 2). In addition to five healthy control samples, we included RNA samples from biopsies of patients affected by un-related mitochondrial myopathies. These patients were previously determined to have heteroplasmic...
mtDNA mutations resulting mainly in deficiencies in mitochondrial complex III and/or complex IV. qRT-PCR confirmed that expression of the mitochondrial iron importer mitoferrin 2 (MFRN2) mRNA was significantly increased by an average of 3.5-fold in the ISCU Myopathy patients (Figure 2A), whereas the microarray data suggested that mitoferrin 1 (MFRN1) expression was decreased by 1.5-fold (Table S1). qRT-PCR also confirmed that FBXL5, the ubiquitin ligase responsible for targeted degradation of IRP1 and IRP2, increased by an average 2.6-fold (Figure 2B). Moreover, two enzymes involved in sulfur metabolism, namely TST (rhodanese) and cysteine desulfurase NFS1, were increased by 4.3- and 3.7-fold in ISCU patient biopsies, respectively (Figure 2C,D). Finally ALAS1, the initial and rate-limiting step in heme biosynthesis, was increased by an average of 4.7-fold in patient biopsies (Figure 2E).

We next tested whether the above iron metabolism genes were also up-regulated during short-term, siRNA-mediated ISCU depletion in cultured primary myoblasts. Depletion of ISCU with siRNA resulted in decreased Fe-S protein levels in three different cellular compartments including mitochondria (FECH), cytosol (PPAT), and nucleus (NTHL1), and also decreased cytosolic aconitase enzyme activity (Figure 2F). Moreover, cellular iron homeostasis was disrupted by ISCU depletion, as assessed by induction of IRP-IRE-binding activities of IRP1 and IRP2, and increased IRP2 and TfR protein levels (Figure 2G). qRT-PCR analysis of gene expression in these cells revealed significant increases in MFRN2 and ALAS1 (Figure 2H), albeit of much smaller magnitude than the increase seen in ISCU patient muscle biopsies; we were unable to observe reproducible changes in FBXL5, TST, or NFS1 levels during ISCU depletion in the myoblasts.
Altered muscle fiber composition and increased capillary perfusion in ISCU Myopathy patient skeletal muscles.

The bioinformatics analyses presented above indicated coordinated changes in expression of myofibril structural components, suggesting that alterations in skeletal muscle fiber composition might be of relevance to the ISCU Myopathy disease phenotype. We examined our microarray dataset more closely and found a relatively large group of down-regulated genes that were annotated as structural components of fast-twitch, glycolytic muscle fibers (Table S2). We also found a smaller group of up-regulated muscle-specific genes that included the cardiac myosin MYH6 (Table S2), which was reported to co-express with slow-twitch MHCI in human facial muscles 100, and in rabbits during fast to slow skeletal muscle fiber-type transition 101. MYH7B, which was previously shown to be expressed in slow-tonic fibers of rat extraocular muscle and in muscle spindle bag fibers 102, was also significantly up-regulated (Table S2). Moreover, over 30 genes encoding components of the mitochondrial respiratory chain were increased significantly, but to a level below our cut-off of 1.5-fold (data not shown).

qRT-PCR analysis confirmed profound decreases in glycolytic muscle fiber contractile motor protein component myosin heavy chain 1 (MYH1; Figure 3A) and the F-actin crosslinking protein α-3 actinin (ACTN3; Figure 3B) in ISCU patient biopsies. The creatine biosynthetic enzyme glycine amidinotransferase was also strongly down-regulated (GATM; Figure 3C). Concomitantly, expression of MYH6 was increased in ISCU patient biopsies (Figure 3D), as was expression of monocarboxylate transporters MCT1 and MCT2 (Figure 3E,F), which have been shown to be highly expressed in human type I oxidative fibers 103. To further explore potential alterations in muscle fiber type in ISCU Myopathy patients, we performed differential myosin
ATPase histochemistry on control and patient biopsy sections according to the method of Dubowitz and Sewry \(^{104}\). This method revealed a significant 22% relative increase in oxidative type I fibers, accompanied by a significantly decreased total fraction of type II glycolytic fibers (Figure 3E). More specifically, type IIa fibers, which show fast shortening speed and the capacity for both aerobic and anaerobic contractile activity, were significantly decreased by 13%, while true type IIb glycolytic fast-twitch fibers decreased non-significantly by 9% (Figure 3E). Together, these methods demonstrated a clear decrease in the number of glycolytic muscle fibers in ISCU Myopathy patient biopsies, and an apparent increase in the relative fraction of oxidative fibers.

A recent clinical study reported increased capillary numbers in skeletal muscle of mitochondrial myopathy patients with mitochondrial respiratory chain defects \(^{105}\). Microarray and qRT-PCR analysis of our ISCU patient muscle biopsies demonstrated significant increases in vascular endothelial growth factor B (VEGFB; Figure 4A), the VEGF receptor KDR (Figure 4B), hypoxia-inducible factor 2α (HIF2A; Figure 4C), and basic fibroblast growth factor (FGF2; Figure 4D) \(^{106}\). These changes in angiogenesis-related gene expression suggested that enhanced capillary formation might accompany the altered muscle fiber type composition in the ISCU Myopathy patients. To investigate this further, we stained muscle sections from an ISCU patient and the healthy heterozygous offspring of this patient with an antibody specific to the capillary endothelial marker protein, CD31. There was a clear increase in the number of capillaries in the patient section as compared to the unaffected heterozygote section (Figure 4E,F).

**Altered energy substrate utilization in ISCU Myopathy patient muscle fibers**
Alterations in muscle energy metabolism remain relatively uncharacterized in ISCU Myopathy patients, despite the known impairments in oxidative phosphorylation attributable to deficiencies in mitochondrial aconitase and succinate dehydrogenase. Moreover, a decrease in the ratio of glycolytic to oxidative muscle fibers as reported above would also likely result in altered expression of energy metabolism genes. Our microarray analysis revealed decreased expression of several key glycolytic enzymes including muscle-specific phosphofructokinase (PFKM), phosphoglycerate kinase 1 (PGK1), aldolase (ALDOA), and muscle-specific enolase (ENO3) (Table 2). Notably, expression of the muscle-specific lactate dehydrogenase isoform LDHA was significantly decreased by -2.1-fold, while the heart LDH isoform LDHB was increased by 3.4-fold (Table 2), suggesting a shift in the relative abundance of LDH isozymes in ISCU Myopathy patient muscles.

Functional profiling of the microarray output data using both IPA and iProXpress suggested significant alterations in PPAR-regulated genes present in pathways involving fatty acid metabolism (Table 1). Several genes in this group were directly involved in cytosolic fatty acid transport (FABP3, 4, and 6), mitochondrial fatty acid uptake (CPT1A, SLC25A20, and CPT2), and mitochondrial and peroxisomal fatty acid β-oxidation (ETFB, EHHADH, ACAA2, ECH1, ACOX2, MCEE, and PCCB) (Table 2). qRT-PCR verified up-regulation of the functional units of the mitochondrial carnitine palmitoyltransferase system, CPT1A, SLC25A20, and CPT2, which were increased by an average of 3-fold, 2.6-fold, and 4.6-fold, respectively (Figure 5A-C). Cytosolic fatty acid binding protein 4 (FABP4), the expression of which was previously shown to be increased in the muscles of endurance-trained athletes, was increased by an average of 5.5-fold (Figure 5D). Interestingly, expression of the mitochondrial uncoupling
protein UCP2, which dissipates the proton gradient formed by the electron transport chain, was also significantly increased by an average of 7.2-fold in ISCU Myopathy patient biopsies (Figure 5E). Finally, we found that quinolinate phosphoribosyltransferase (QPRT), a key enzyme in the pathway for de-novo synthesis of nicotinamide adenine dinucleotide (NAD+) \(^{109}\), was up-regulated by an average of 16.8-fold in ISCU patient muscle biopsies (Figure 5F), possibly indicating increased NAD+ production in ISCU Myopathy patient muscles.

**Evidence for ketone body formation in ISCU Myopathy patient muscles**

  Ketogenesis occurs in mammals in response to organism-level energy starvation \(^{110}\). Activation of ketogenesis results in elevated circulating levels of the ketone bodies acetoacetate, acetone, and (R)-3-hydroxybutyrate. Our microarray data suggested that the rate-limiting mitochondrial enzyme involved in ketone body formation, HMGCS2, was up-regulated in ISCU Myopathy patient muscles (Table 2). In fact, HMGCS2 was the most highly up-regulated gene in the dataset. Accordingly, qRT-PCR verified an average 27-fold induction of HMGCS2 in the ISCU Myopathy patients (Figure 6A), and elevated HMGCS2 expression was found in one of the non-ISCU mitochondrial myopathy patients as well (Figure 6A). Expression of 3-hydroxybutyrate dehydrogenase (BDH1), which interconverts the two major ketone body species 3-hydroxybutyrate and acetoacetate, was also increased by an average of 3.2-fold in ISCU Myopathy patient biopsies (Figure 6B). In addition to inducing ketogenesis, long-term energy starvation also has been shown to up-regulate hepatic expression of the secreted endocrine hormone FGF-21 \(^{111}\). Our microarray data showed that muscle FGF-21 expression was significantly increased (Table 2), and qRT-PCR analysis confirmed an average 25-fold induction
of FGF21 mRNA in ISCU Myopathy patient muscle biopsies (Figure 6C). Together, these results suggest that impaired energy metabolism in ISCU Myopathy patient muscles leads to up-regulation of ketogenic enzymes and synthesis of a starvation-induced hormone in skeletal muscle.

To verify the dramatic increases that we observed in HMGCS2 mRNA expression in the ISCU Myopathy patient muscle biopsies, we performed northern blots using total RNA extracts derived from control and patient muscle biopsies. Northern blots confirmed that HMGCS2 was dramatically up-regulated in ISCU Myopathy patient biopsies, and also illustrated a high degree of HMGCS2 expression in one of the non-ISCU Myopathy patients (Figure 6D). We next asked whether HMGCS2 mRNA expression occurred in other ISCU Myopathy patient cell types. We found that HMGCS2 mRNA expression was restricted to the patient muscle biopsies, and was absent in control and patient myoblasts, fibroblasts and lymphoblasts (Figure 6E), despite varying degrees of ISCU depletion in these cell lines (Crooks et al., manuscript in preparation). These data suggest that aberrant HMGCS2 expression occurs only in mature skeletal muscle fibers in ISCU Myopathy patients.

The observation of elevated mRNA expression of ketogenic enzymes and the secreted hormone FGF-21 in ISCU Myopathy patient biopsies suggested that signs of these changes might be evident in the systemic circulation of these patients. Therefore, we measured the concentrations of the most prevalent ketone body (3-hydroxybutyrate), and FGF21 in patient plasma samples. We obtained two separate plasma samples per patient, taken several days apart from each other, and we included plasma samples from five un-related mitochondrial myopathy patients and five healthy controls in the analysis as well. The results of the ketones test showed
mildly elevated 3-hydroxybutyrate levels in both patients during the day of the first blood draw (Figure 6F). However, these 3-hydroxybutyrate concentrations were still well within the normal range of plasma ketone levels when the variables of diet composition and time of fasting before the blood draw were taken into consideration. However, plasma FGF21 levels were found to be significantly elevated in both of the ISCU Myopathy patients, and the FGF21 levels in three of the non-ISCU Myopathy patients were also much higher than the controls (Figure 6G). Together, these data demonstrate that elevated plasma FGF21 expression may be useful as a biomarker of impaired oxidative metabolism in ISCU Myopathy patient skeletal muscles.
Discussion

Skeletal muscle plays a critical role in organism-level energy metabolism, and it is unique in its capacity to adapt to the energy demands of spontaneous exercise. ISCU protein deficiency in the skeletal muscle of ISCU Myopathy patients causes profound decreases in key mitochondrial Fe-S enzymes including succinate dehydrogenase and aconitase, leading to lifelong exercise intolerance and sporadic periods of severe weakness and rhabdomyolysis. We hypothesized that the pathophysiological aspects of the ISCU Myopathy disease phenotype would be reflected by alterations in the underlying gene expression response. Indeed, we observed an up-regulation of several key iron metabolism genes in this study, but in addition we identified modifications in muscle fiber composition and energy utilization pathways. Paradoxically, increased oxidative muscle fibers and fatty acid metabolism gene expression was seen in ISCU Myopathy patient muscles despite the well-known TCA cycle enzyme deficiencies. The preference for fatty acids as an energy source was also associated with a strong induction of the rate-limiting ketogenic enzyme HMGCS2 and the starvation response hormone FGF21, leading to greatly elevated plasma FGF21 levels. These results illustrate striking morphological and metabolic remodeling in skeletal muscle as a consequence of chronic ISCU deficiency, and suggest that elevated plasma FGF21 may be a clinically relevant biomarker for the diagnosis and treatment of ISCU Myopathy and other mitochondrial myopathies as well.
Altered Iron Metabolism in ISCU Myopathy

Studies describing the effect of long-term Fe-S cluster assembly deficiency on mammalian cellular iron metabolism have been limited to investigations of Friedreich’s Ataxia models \(^{112-114}\), and cells derived from a sideroblastic anemia patient with GLRX5 deficiency \(^{115}\). In the present study we observed increased expression of the sulfur metabolism genes NFS1 and rhodanese (TST). In contrast to this, Tan et al (2003) reported decreased NFS1 and rhodanese in cultured frataxin patient cells \(^{114}\), while Huang et al. (2009) also reported decreased NFS1 expression in cardiac tissue of frataxin conditional knockout mice \(^{112}\). These differences suggest that iron metabolism gene expression patterns ISCU Myopathy are distinct from those observed in frataxin-deficient muscle. Our observation of increased MFRN2 expression, however, is in line with the findings of Huang et al., and suggests that increased mitochondrial iron uptake may contribute to the mitochondrial iron overload seen in affected ISCU Myopathy and Friedreich’s ataxia tissues \(^{112}\).

Mitochondrial energy metabolism relies heavily upon Fe-S- and heme-containing enzymes, which in turn depend on the biosynthetic pathways required for de-novo synthesis of these iron-containing cofactors \(^{116}\). Since ferrochelatase, the terminal heme biosynthetic enzyme, requires Fe-S clusters for stability, ISCU deficiency can negatively impact both Fe-S cluster assembly and heme biosynthesis \(^{78}\). Here, we observed decreased abundance of Fe-S cluster enzymes in three distinct cellular compartments following siRNA-mediated ISCU depletion in primary myoblasts, a finding that is consistent with other studies on depletion of Fe-S cluster assembly components \(^{84,115}\). Our observations of increased expression of the mitochondrial iron
import gene MFRN2 and the rate-limiting mitochondrial heme biosynthetic enzyme ALAS1 during ISCU depletion may reflect a cellular response to (1) depletion of Fe-S clusters due to ISCU deficiency; and (2) decreased heme biosynthesis due to the downstream effect of ferrochelatase depletion. Moreover, the activation of ALAS1 might be the result of transcriptional stimulation by peroxisome proliferator-activated receptor-gamma co-activator 1α (PGC-1α), an effect that has been observed previously in mouse liver during starvation or PGC1α over-expression 117.

Metabolic remodeling of energy metabolism and muscle fiber composition

In light of the marked impairment in mitochondrial oxidative phosphorylation in the muscles of ISCU Myopathy patients, we were surprised to find evidence for enhanced fatty acid oxidation enzymes, capillary perfusion, and increased numbers of oxidative muscle fibers in these patients. Increased formation of oxidative fibers has been seen following endurance exercise in rodents and in marathon runners 118,119, and this fiber type shift has been associated with increased muscle fiber capillary perfusion as well 120,121. Furthermore, histochemical 122, biochemical 123, and proteomic approaches 124 have demonstrated transition from glycolytic to oxidative fibers during chronic electrical stimulation of muscles. We surmise that similar signals within ISCU Myopathy patient muscles might elicit changes in fiber type and capillary numbers in the absence of exercise training.

A number of studies have implicated (PGC1α) as a positive regulator of slow twitch muscle fiber and capillary formation during exercise and other stimuli (reviewed in 120). For example, oxidative muscle fiber formation was observed when PGC1α was over-expressed in
mouse muscles\textsuperscript{125}, and muscle PGC1α levels were increased in rat muscle following exercise stimulation\textsuperscript{126}. This PGC1α-induced fiber type transition was recently linked directly to upregulation of HIF2α\textsuperscript{127}. Consistent with this, we observed a significant up-regulation of HIF2α (EPAS1) mRNA in our ISCU Myopathy patient muscle biopsies (Figure 4C), an effect that likely contributed to the increased capillary perfusion observed here, as well as in other mitochondrial myopathy patients with cytochrome oxidase-deficient ‘ragged red’ fibers\textsuperscript{105}.

We hypothesize that PGC1α activation in ISCU Myopathy patient muscles might be caused by stimuli such as oxidative stress or energy depletion, eventually leading to our observation of increased oxidative fibers and fatty acid metabolism genes. PGC1α can be activated by H$_2$O$_2$\textsuperscript{128} or by sirtuin 1 (SIRT1) via de-acetylation of critical PGC1α lysines\textsuperscript{129}. SIRT1 activity is stimulated during times of starvation by increased NAD+ levels\textsuperscript{129,130}, a condition that likely develops in ISCU Myopathy patient muscles due to Fe-S enzyme deficiency. Indeed, the high pyruvate levels and anomalously low lactate:pyruvate ratios in the circulation of ISCU Myopathy patients strongly suggest a decreased muscle NADH/NAD+ ratio\textsuperscript{59,67}. Our observation of greatly enhanced QPRT expression in patient muscles (Figure 5F) provides further evidence for activation of SIRT1 by NAD+, since QPRT has been suggested to be a rate-limiting enzyme of de-novo NAD+ biosynthesis\textsuperscript{109}. Ultimately, the activation of PGC1α in patient muscle tissue would lead to stimulated expression of PPARα target genes\textsuperscript{131}, including the components of the carnitine palmitoyltransferase system\textsuperscript{132}, the peroxisomal and mitochondrial β-oxidation systems\textsuperscript{133,134}, HMGCS2\textsuperscript{135}, and FGF-21\textsuperscript{136}, all of which we observed in ISCU Myopathy patient skeletal muscle tissue.
Activation of ketogenesis in ISCU Myopathy patient muscles

One problem of enhanced fatty acid oxidation in a situation where TCA cycle enzymes are impaired is how to effectively metabolize the products of this process, namely NADH, FADH$_2$, and acetyl-CoA. Most NADH is oxidized to NAD$^+$ by NADH dehydrogenase (complex I), while the electrons from the FADH$_2$ cofactor of the electron-transferring flavoprotein are donated to complexes III and IV, all of which show relatively mildly impaired activity in ISCU Myopathy patient muscles$^{67}$. However, utilization of acetyl-CoA for ATP production requires its entry into the TCA cycle via condensation with oxaloacetate to form citrate, and further metabolism is dependent on the activities of aconitase and succinate dehydrogenase (complex II), which are dramatically reduced in ISCU Myopathy patient skeletal muscles$^{63,67}$. Therefore, we may expect significant accumulation of mitochondrial acetyl-CoA in ISCU Myopathy patient muscles, which can lead to deleterious allosteric effects on critical enzymes such as pyruvate dehydrogenase and pyruvate carboxylase$^{137}$. Moreover, excess acetyl-CoA accumulation would decrease the pool of free coenzyme A available for thioester activation of other metabolic intermediates such as succinyl-CoA, leading to further metabolic impairments.

Activation of ketogenesis in ISCU Myopathy patient muscles provides a potential mechanism to dispose of excess mitochondrial acetyl-CoA derived from fatty acid β-oxidation, when entry of acetyl-CoA into the TCA cycle is inhibited (Figure 7). Ketogenesis is a process by which two molecules of acetyl-CoA in the mitochondrial are condensed to form the ketone bodies acetoacetate and D-β-hydroxybutyrate$^{110}$. This process is normally initiated in animals during times of glucose deprivation to make the energy-rich products of fatty acid oxidation in the liver available to distally-located tissues such as the brain$^{138}$. We believe that the dramatic
increase in the rate-limiting ketogenic enzyme HMGCS2 that we observed in ISCU Myopathy patient muscles (Figure 6) resulted from a compensatory cellular effort to maintain adequate levels of free coenzyme A in mitochondria during fatty acid oxidation. The resulting ketone bodies would be excreted from the affected muscle fibers and metabolized in unimpaired patient tissues such as brain or liver (Figure 7). Although we did not observe clinically significant elevations in plasma ketones in the ISCU Myopathy patients (Figure 6), the extremely rapid kinetics of plasma ketone clearance by the kidneys and other organs may have precluded us from observing a steady-state increase \[^{139}\].

**Plasma FGF21 as a clinically relevant biomarker of ISCU Myopathy**

In addition to activating fatty acid catabolism and ketogenesis, cellular energy starvation strongly induces expression of the endocrine hormone FGF-21 \[^{111,136}\]. The induction of FGF-21 is thought to regulate the metabolic response to energy deprivation on the organismal level \[^{140}\], and elevated levels of FGF-21 in the blood are induced by hepatic PPAR\(\alpha\) activation during fasting \[^{111}\]. The effects of elevated plasma FGF-21 include reduced plasma glucose and triglyceride levels \[^{141}\], as well as enhanced liver fatty acid oxidation and ketogenesis \[^{136}\]. Interestingly, HMGCS2 over-expression has been shown to increase FGF-21 mRNA expression \[^{142}\], while FGF-21 over-expression increased HMGCS2 protein levels \[^{111}\], suggesting that these genes may regulate each other in a feed-forward mechanism in ISCU Myopathy patient muscles.

Furthermore, a recent study proposed an autocrine/paracrine function for FGF-21 secreted by adipocytes during prolonged cold exposure that resulted in elevated adipocyte PGC1\(\alpha\) protein levels \[^{143}\].
Given that other tissues in the ISCU Myopathy patients appear clinically normal and express higher levels of ISCU protein\textsuperscript{73} (Crooks et al., in preparation), the elevated plasma FGF-21 in our patients is likely the result of FGF-21 secretion directly from affected skeletal muscles. This is not unprecedented, as elevated FGF-21 expression in skeletal muscle has been reported in response to hyperinsulinemia\textsuperscript{144,145}, and in a mouse model of late-onset mitochondrial myopathy\textsuperscript{146,147}. Importantly, elevated FGF-21 mRNA expression in this mouse model was not observed in any tissue other than skeletal muscle\textsuperscript{147}. A recent retrospective clinical study by Suomalainen et al. identified elevated plasma FGF-21 level as a strongly positive indicator for muscle-manifesting mitochondrial diseases\textsuperscript{148}. The elevated plasma FGF-21 concentrations that we measured in our ISCU Myopathy patients (1366-2979 pg/mL) and in the non-ISCU mitochondrial myopathy patients (573-2749 pg/mL) were in the range of the FGF-21 levels reported in other moderate to severe mitochondrial myopathy patients\textsuperscript{148}. Thus, plasma FGF-21 may prove to be useful in the diagnosis and treatment of ISCU Myopathy.

In summary, we have used gene expression analysis to explore the unique pathophysiology of a human mitochondrial myopathy caused by deficiency of Fe-S clusters. Functional analysis revealed alteration of several key pathways involved in muscle fiber composition, enhanced fatty acid metabolism, and ketogenesis. Expression of the starvation response hormone FGF-21 in affected patient muscle fibers likely gave rise to the highly elevated FGF-21 levels measured in patient plasma, suggesting that this clinically-relevant biomarker of mitochondrial disease may be useful both in diagnosis, and in evaluating the efficacy of therapeutic treatments for this and other mitochondrial myopathies.
Materials and Methods

Patients, Tissue Biopsies and Primary Cultures

Three ISCU Myopathy patients were included in this study, aged 42, 42, and 66 years, as well as one 43 year old unaffected heterozygous individual. Controls consisted of five healthy individuals, ages 22-47 years. Five patients of ages 29-63 years affected by un-related mitochondrial myopathy due to heteroplasmic mitochondrial DNA mutations were also included in some analyses. These patients showed a peak systemic arterio-venous oxygen (a-vO$_2$) difference of 4.5-8.3 mL/dL during maximal exercise, compared with an average peak a-vO$_2$ difference of ~5 mL/dL in the ISCU Myopathy patients, and ~15 mL/dL in healthy controls. Skeletal muscle biopsies from *vastus lateralis* were obtained and prepared as described previously $^{15}$. Written informed consent was given by all individuals involved in the study in accordance with the Institutional Review Board of the University of Texas Southwestern Medical Center and the Presbyterian Hospital of Dallas. Primary myoblast cell lines, a generous gift from Dr. Eric Shoubridge, were cultured as described previously $^{48}$, except that Lonza SKBM-2 culture medium was used. Primary fibroblasts were cultured in DMEM medium containing 5mM glucose and 110mg/L sodium pyruvate and supplemented with 10% FBS, and lymphoblasts were cultured in RPMI 1640 buffered with 25mM HEPES and supplemented with 10% FBS.

RNA Preparation and Microarray Analysis

RNA from biopsies and cell pellets was prepared using the mirVana™ RNA Isolation Kit (Ambion) according to the manufacturer’s instructions. RNA quality was verified using an
Agilent 2100 Bioanalyzer, and samples were processed by 1-cycle target labeling and hybridization with Affymetrix Genechip U133 Plus 2.0 microarrays at the NCI-Frederick Laboratory of Molecular Technology. R/Bioconductor software was used to identify differentially expressed genes in the dataset. The Robust Multi-Array Average expression measure (RMA) was applied to perform background correction, quantile normalization and finally summarization of expression values in log2 scale. Normalized unscaled standard error (NUSE) and relative log expression (RLE) plots were generated for quality assessment. We used the LIMMA package to assess differentially expressed genes in the ISCU Myopathy biopsy samples. Tests for significance were calculated and adjusted for multiple testing by controlling the Benjamini and Hochberg’s false discovery rate at 5%.

The hgu133plus2.db, GO.db and KEGG.db packages were used to annotate the differentially expressed genes. Functional and pathway analyses were performed using iProExpress, DAVID, Ingenuity Pathway Analysis™ software, and Genomatix™.

**Northern Blots and qRT-PCR Analysis**

qRT-PCR was performed using SYBR® Green (Applied Biosystems) according to the manufacturer’s instructions, following reverse transcription of total RNA into cDNA (Applied Biosystems). qRT-PCR primer sequences are listed in Table S3. Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal control. Correct qRT-PCR product size was verified by agarose gel electrophoresis, and automated melting curves were generated and assessed after every experiment. Northern blots were performed as reported previously. An antisense RNA probe against HMGCS2 mRNA was synthesized using the
MAXIScript T7 *in vitro* transcription kit (Ambion) incorporating $\alpha^{32}$P-cytosine triphosphate. The probe template with T7 promoter was generated by PCR using a human liver cDNA library and the following primers: 5’-TAATACGACTCACTATAGGGAGACCATAAGAGAAGGCACCAATCC-3’ and 5’-TCCCCCTGGCCAAAACAGA-3’.

**siRNA knockdown of ISCU**

A pool of siRNA oligonucleotides directed against ISCU was purchased from Thermo Scientific / Dharmacon (part# L-012837-01-0005); a pool of non-targeting oligonucleotides served as the control. Primary myoblasts were transfected with 100nM siRNA using the Neon™ Transfection System (Invitrogen), with settings of 1400 V, 20 ms pulse width, and 2 pulses per transfection. The cells were transfected every other day for six days, and were harvested two days after the last transfection.

**Muscle Fiber Type Quantification and CD31 histochemistry**

Muscle fiber composition was assessed by acid-base myofibrillar ATPase staining $^{104}$. Biopsies from three different ISCU Myopathy patients were included in the experiment, and 13 healthy control biopsies were included for comparison. Immunohistochemistry for the capillary endothelial marker CD31 was performed on one patient muscle biopsy, and one biopsy from the healthy heterozygous offspring of this patient, as previously described $^{105}$.

**Western Blots**
SDS-PAGE and western blotting were performed as described previously \(^4^4\) using 1.5mm 4-12\% pre-cast bis-Tris gels (Invitrogen). Rabbit polyclonal anti-ferrochelatase serum was a kind gift from Dr. Harry Dailey. Anti-IRP2 and ISCU rabbit polyclonal sera were raised against synthetic peptide fragments \(^7^9\). Mouse monoclonal anti-NTHL1 was from R&D Systems. Mouse monoclonal anti-human TfR1 was from Zymed.

**Aconitase In-Gel Assay and Electrophoretic Mobility Shift Assay (EMSA)**

Aconitase was assayed using a coupled assay following native PAGE separation, as described previously \(^4^6\). IRP-IRE binding activity was determined by EMSA using a \(^3^2\)P-labeled ferritin IRE probe, as described previously \(^4^6\).
Figure 1: Global Gene Expression Analysis In ISCU Myopathy Patients. Informatics tools were used to identify cellular structures and processes that were affected in ISCU Myopathy patient biopsies based on microarray gene expression analysis. (A) The DAVID bioinformatics tool was used to identify sub-cellular structures in which organelle-specific gene clusters were significantly altered in patient biopsies. \( P<0.001 \) for all groups shown. (B) Gene clusters representing cellular processes that were significantly altered in patient muscle biopsies were identified using Ingenuity Pathway Analysis. At least 20% of the genes in each functional group shown were significantly up- or down-regulated.
## Table 1

### GO Slim Statistics (Process)

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<td>GO:0006939</td>
<td>Muscle contraction</td>
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<td>GO:0007005</td>
<td>Mitochondrion organization</td>
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### KEGG Pathway Statistics

| PATH:hsa00010 | Glycolysis/Gluconeogenesis                    | 11             | 13           | 24    |
| PATH:hsa00020 | Citrate cycle/TCA cycle                       | 2              | 11           | 13    |
| PATH:hsa00061 | Fatty acid biosynthesis                       | 0              | 1            | 1     |
| PATH:hsa00071 | Fatty acid metabolism                         | 3              | 17           | 20    |
| PATH:hsa00072 | Synthesis and degradation of ketone bodies    | 0              | 3            | 3     |
| PATH:hsa00190 | Oxidative phosphorylation                     | 5              | 36           | 41    |
| PATH:hsa00280 | Valine Leucine Isoleucine degradation         | 2              | 18           | 20    |
| PATH:hsa00290 | Valine Leucine Isoleucine biosynthesis        | 1              | 3            | 4     |
| PATH:hsa00480 | Glutathione metabolism                       | 4              | 7            | 11    |
| PATH:hsa04370 | VEGF signaling                               | 16             | 10           | 26    |
| PATH:hsa00650 | Butanoate metabolism                         | 1              | 10           | 11    |
| PATH:hsa03320 | PPAR signaling                               | 5              | 23           | 28    |
| PATH:hsa00010 | Graft versus host disease                    | 0              | 17           | 17    |

Table 1

Functional profiling of gene expression data using iProXpress based on GO Process and KEGG pathway terms
Figure 2

Figure 2: ISCU deficiency in ISCU Myopathy patient biopsies and in ISCU-depleted myoblasts alters expression of several iron metabolism genes. qRT-PCR analysis of mRNA isolated from vastus lateralis biopsies from five healthy control individuals, three ISCU Myopathy patients, the healthy heterozygous offspring of an ISCU Myopathy patient, and five individuals with unrelated mitochondrial myopathies. ISCU Myopathy biopsies showed significant increases in mRNA expression of (A) Mitoferrin 2 (MFRN2; average 3.5-fold) (B) F-box and leucine-rich repeat protein 5 (FBXL5; average 2.6-fold) (C) rhodanese (TST; average 4.3-fold) (D) cysteine desulfurase (NFS1; 3.7-fold), and (E) δ-aminolevulinic acid synthase (ALAS1; 4.7-fold). The non-transformed $2^{-\Delta\Delta Ct}$ values were analyzed by one-way ANOVA with
Holm-Sidak post-hoc test to determine statistical significance. ** $P<0.01$ (F) siRNA depletion of ISCU protein led to decreased ferrochelatase (FECH), glutamine phosphoribosylpyrophosphate amidotransferase (PPAT), and DNA glycosylase NTHL1 protein levels, as well as decreased mitochondrial (mACO) and cytosolic (cACO) aconitase activities. (G) Electrophoretic mobility shift assay of whole-cell lysates using a $^{32}$P-labeled ferritin IRE probe demonstrated activation of iron regulatory protein 1 (IRP1), and western blots showed increased levels of IRP2 and transferrin receptor (TfR) proteins. (H) qRT-PCR analysis of control and ISCU-depleted myoblasts demonstrated that decreased ISCU expression levels were accompanied by significant increases in MFRN2 and ALAS1 mRNA expression levels, while FBXL5, NFS1 and TST expression levels were not significantly changed. Data were analyzed by 2-tailed Student’s t-test (n=3). *$P<0.05$; **$P<0.01$. Similar effects were observed in three separate experiments.
Figure 3: A shift from glycolytic to oxidative muscle fibers in ISCU Myopathy patients. qRT-PCR demonstrated decreased expression of type II muscle fiber contractile protein myosin heavy chain 1 (A; MYH1; average 0.25-fold), the structural protein α-3 actinin (B; average 0.21-fold), and the creatine biosynthetic enzyme glycine amidinotransferase (C; average 0.13-fold). (D) Cardiac myosin MYH6 was increased by an average of 3.8-fold in ISCU Myopathy patient biopsies. (E) Muscle fiber composition in ISCU Myopathy patient and control biopsy sections was quantified histochemically by differential myosin-ATPase staining (see Materials and Methods). The number of type I fibers was significantly higher in ISCU Myopathy patients (n=3) as compared to controls (n=13), while the number of type IIa fibers was significantly lower, and the number of type IIb fibers trended lower. Two-tailed, unpaired t-tests were used to determine statistical significance. *P<0.05; P<0.01.
Figure 4: Increased expression of angiogenesis-related genes and enhanced capillary perfusion in ISCU Myopathy patient muscles. qRT-PCR revealed significant increases in (A) vascular endothelial growth factor B (VEGFB; average 2.7-fold), (B) the VEGF receptor KDR (average 4-fold), (C) hypoxia-inducible factor 2α (HIF2α/EPAS1; average 3.3-fold), and (D) fibroblast growth factor 2 (FGF2; average 9-fold) in ISCU Myopathy patient biopsies. The non-transformed $2^{-\Delta\Delta C_t}$ values were analyzed by one-way ANOVA with Holm-Sidak post-hoc test. * $P<0.05$; ** $P<0.01$. (E,F) Immunohistochemistry demonstrated an increased abundance of CD31-positive capillaries perfusing the interstitial space between myofibers in muscle sections taken from an ISCU Myopathy patient (F), as compared to the healthy heterozygous offspring of this individual (E).
### Table 2

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**Table 2:** List of genes related to energy metabolism that were significantly changed in the microarray dataset
Figure 5: Enhanced mitochondrial fatty acid uptake and metabolism in ISCU Myopathy patient muscles. The pattern of increases in fatty acid metabolism genes identified by Ingenuity Pathway Analysis was confirmed by qRT-PCR analysis of genes involved in mitochondrial fatty acid uptake. (A) Liver-specific carnitine palmitoyltransferase I (CPT1A) was increased in two of the three ISCU Myopathy patients evaluated. (B) The acyl-carnitine translocase SLC28A20 was significantly increased by an average of 4.5-fold in ISCU Myopathy patients. (C) Carnitine palmitoyltransferase II (CPT2) was significantly increased by an average of 4.6-fold. (D) Cytosolic fatty acid binding protein 4 (FABP4) was increased by an average of 5.5-fold in ISCU Myopathy patients. (E) Mitochondrial uncoupling protein 2 (UCP2) was increased by an average of 7.2-fold in ISCU Myopathy patients and 2.7-fold in the Mitochondrial Myopathy patients. (F) Quinolinate phosphoribosyltransferase (QPRT) was elevated by an average of 16.8-fold in ISCU Myopathy patient biopsies.
Figure 6: Upregulation of the ketogenic pathway in ISCU Myopathy patient muscles. The ketogenic enzymes hydroxymethylglutaryl coenzyme-A synthase 2 (A; HMGCS2) and β-hydroxybutyrate dehydrogenase 1 (B; BHD1) were increased in ISCU Myopathy patient
biopsies. (C) The starvation-response hormone fibroblast growth factor 21 (FGF21) was significantly increased in ISCU Myopathy patient biopsies, and was also elevated to varying degrees in all of the Mitochondrial Myopathy patients. (D) The increase in HMGCS2 observed by qRT-PCR was confirmed by northern blot using a $^{32}$P-labeled RNA probe synthesized from a fragment of the HMGCS2 gene (see Materials and Methods). (E) HMGCS2 mRNA expression levels were evaluated by northern blot in skeletal muscle biopsies, primary myoblasts, fibroblasts, and lymphoblasts derived from ISCU Myopathy patients and healthy controls. (F) Plasma β-hydroxybutyrate was measured enzymatically. (G) FGF21 concentration was measured in plasma by ELISA (See Materials and Methods)
Figure 7: Morphological and biochemical alterations caused by ISCU depletion in human skeletal muscle. A schematic diagram summarizing the morphological differences between (A) control, and (B) ISCU Myopathy patient muscle biopsies, as inferred from gene expression analysis, histochemical data, and previous studies. ISCU Myopathy patient muscle biopsies showed decreased numbers of glycolytic myofibers and increased numbers of oxidative myofibers, as well as increased capillary perfusion. Mitochondrial proliferation is inferred from previous reports of significantly increased citrate synthase activities in patient muscle. (C) Biochemical alterations in ISCU Myopathy patient muscles due to prolonged ISCU depletion and TCA cycle deficits include enhanced mitochondrial fatty acid uptake capacity due to increased expression of the components of the carnitine palmitoyltransferase system (CPT1A, SLC28A25, and CPTII) and increased expression of mitochondrial and peroxisomal fatty acid β-oxidation genes. We propose that deficits in aconitase and succinate dehydrogenase lead to a buildup of acetyl-Coenzyme A (acetyl-CoA); Ketogenesis is activated by up-regulation of the rate-limiting ketogenic enzyme HMGCS2 in order to dispose of the excess acetyl-CoA as ketone bodies and liberate free coenzyme A. These alterations in gene expression are likely driven by transcriptional activation of PPARα and PGC1α, which are activated by low ATP levels and high NAD+ levels. The metabolic deficit is also accompanied by increased myofiber expression and
secretion of the starvation-induced hormone FGF-21, leading to measurable FGF-21 increases in patient plasma.

**Table S1**

<table>
<thead>
<tr>
<th>Genes Involved in Iron Regulation</th>
<th>Gene Symbol</th>
<th>Fold-Change</th>
<th>p-value</th>
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<tr>
<td>Aminolevulinate, delta-, synthase 1</td>
<td>ALAS1</td>
<td>2.0</td>
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<tr>
<td>Thiosulfate sulfurtransferase (rhodanese)</td>
<td>TST</td>
<td>1.9</td>
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<tr>
<td>F-box/LRR-repeat protein 5</td>
<td>FBXL5</td>
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<tr>
<td>Mitoferrin-2</td>
<td>SLC25A28</td>
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<tr>
<td>Metalloreductase STEAP4</td>
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<td>0.005</td>
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<tr>
<td>Ferritin heavy chain</td>
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<td>0.029</td>
</tr>
<tr>
<td>Mitoferrin-1</td>
<td>SLC25A37</td>
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<td>0.014</td>
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<td>Iron-sulfur cluster assembly enzyme ISCU</td>
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<td>0</td>
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<tr>
<td>Slow-twitch Muscle Fiber Genes</td>
<td>Gene Symbol</td>
<td>Fold-Change</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>myosin, heavy chain 6, cardiac muscle, alpha</td>
<td>MYH6</td>
<td>2.6</td>
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<tr>
<td>cytochrome c oxidase subunit IV isoform 1</td>
<td>COX4I1</td>
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<tr>
<td>myosin, heavy chain 7B, cardiac muscle, beta</td>
<td>MYH7B</td>
<td>1.5</td>
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<tr>
<td>cofilin 2 (muscle)</td>
<td>CFL2</td>
<td>-1.7</td>
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<table>
<thead>
<tr>
<th>Fast-twitch Muscle Fiber Genes</th>
<th>Gene Symbol</th>
<th>Fold-Change</th>
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<tbody>
<tr>
<td>tropomyosin 1 (alpha)</td>
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<tr>
<td>sarcolipin</td>
<td>SLN</td>
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</tr>
<tr>
<td>tribbles homolog 1 (Drosophila)</td>
<td>TRIB1</td>
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<tr>
<td>four and a half LIM domains 1</td>
<td>FHL1</td>
<td>-1.8</td>
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<td>myosin, heavy chain 2, skeletal muscle, adult</td>
<td>MYH2</td>
<td>-1.8</td>
</tr>
<tr>
<td>troponin C type 2 (fast)</td>
<td>TNNC2</td>
<td>-1.9</td>
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<tr>
<td>myomesin (M-protein) 2, 165kDa</td>
<td>MYOM2</td>
<td>-1.9</td>
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<td>homer homolog 1 (Drosophila)</td>
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<td>myozin 3</td>
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<tr>
<td>troponin T type 3 (skeletal, fast)</td>
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<td>troponin I type 2 (skeletal, fast)</td>
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<td>myosin binding protein C, fast type</td>
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<td>ATPase, Ca++ transporting, cardiac muscle, fast twitch 1</td>
<td>ATP2A1</td>
<td>-3.8</td>
</tr>
<tr>
<td>smoothelin-like 1</td>
<td>SMTNL1</td>
<td>-5.0</td>
</tr>
<tr>
<td>ankyrin repeat domain 2 (stretch responsive muscle)</td>
<td>ANKRD2</td>
<td>-5.7</td>
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<tr>
<td>myosin, heavy chain 1, skeletal muscle, adult</td>
<td>MYH1</td>
<td>-7.7</td>
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<tr>
<td>actinin, alpha 3</td>
<td>ACTN3</td>
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CONCLUSIONS

In this collection of studies we have examined novel ways in which Fe-S clusters impact cellular developmental processes and human health. We characterized a direct connection between the processes of Fe-S assembly and heme biosynthesis that influences the transformation of immature erythroblasts into circulating red blood cells. We identified novel mechanisms by which Fe-S cluster deficiency can manifest in a single human tissue type, while other tissues remain unharmed. And finally, we identified previously unknown pathways that are activated in Fe-S cluster-deficient human skeletal muscle tissue and discovered a novel disease biomarker that may be useful in the clinic. Together, these studies demonstrate that these ancient Fe-S cluster cofactors still have much to reveal about the nature of our physiology and their impact on our health.

The connection between Fe-S cluster assembly and heme biosynthesis

The first evidence for the interconnected nature of Fe-S clusters and heme biosynthesis came in 1991, when an IRE element was discovered in the 5' UTR of the mRNA encoding erythroid-specific δ-aminolevulinic acid synthase (ALAS2). ALAS2 catalyzes the initial step in heme biosynthesis, and is expressed in developing erythroid cells. The implications were that the initiation of heme biosynthesis could be impaired by the inability of IRP1 to ligate its Fe-S cluster, which would lead to activation of its IRE-binding activity and translational repression at the ALAS2 mRNA. The subsequent discovery that ferrochelatase requires an Fe-S cluster for enzymatic activity raised the possibility that Fe-S clusters might affect heme biosynthesis at the terminal step as well as the initial step. However, little attention was paid to
this possibility in reports written after 1994. Our study expanded on the role of the Fe-S cluster of ferrochelatase by identifying physiologically relevant situations in which iron availability and Fe-S cluster assembly strongly influence the cellular fate of ferrochelatase. We can now conclude that both the first and last steps of heme biosynthesis are intimately, yet distinctly linked to successful assembly of Fe-S clusters.

**Tissue specificity and pathophysiology of ISCU Myopathy**

Our studies on ISCU Myopathy patients were initiated several years ago in hopes of gaining insight into mechanisms that regulate mitochondrial iron homeostasis. However, we quickly realized that several other fundamental questions about this unique disease merited our consideration. Why is the clinical phenotype restricted to skeletal muscle tissue? What compensatory responses are initiated in a tissue that shows such a profound loss of Fe-S clusters? We soon discovered that primary cells derived from these patients did not show the same degree of decrease in ISCU protein levels as did the patient muscle biopsies, and at the same time published reports were appearing reporting similar findings. Our study builds on these findings by demonstrating more complete pathological splicing of ISCU mRNA in cells that have been engineered to overexpress the muscle-specific transcription factor, MyoD. Furthermore, we showed that ROS generated in skeletal muscle may further exacerbate the depletion of ISCU in patient skeletal muscle. The mechanism by which oxidative stress causes rapid depletion of ISCU protein in cells merits further investigation.

We discovered several active biochemical and developmental pathways in ISCU Myopathy patient muscles that have not been previously associated with the disease. Skeletal
muscle hypertrophy, especially in the calf muscles, has been known to be present in these patients since the first report in 1964. We believe that the transition from glycolytic to oxidative muscle fiber types and the increased capillary perfusion that we have identified in these patients can help to explain this hypertrophic physical phenotype. Activation of these pathways is likely a result of a prolonged energy starvation response, mediated by PGC-1α, which causes proliferation of the muscle fibers. The same starvation response also appears to underlie the enhanced expression of fatty acid metabolism genes and the activation of ketogenesis. Our results are in line with a recent report on the metabolic remodeling that occurred in a mouse model of mitochondrial myopathy, and together, our findings have shed new light on the molecular pathophysiology of ISCU Myopathy.

**Potential diagnostics and therapy for ISCU Myopathy patients**

Our discovery that plasma FGF-21 concentrations are greatly elevated in the ISCU Myopathy patients has several implications for future diagnosis and treatment of this disease. Currently, the two ‘gold standards’ for assessing muscle function in mitochondrial myopathies are muscle punch biopsy for biochemical and histological assessment, and maximal exercise testing on a cycle ergometer. Both of these diagnostic methods have major shortcomings in terms of safety and patient comfort. Muscle biopsy is a painful procedure requiring minor surgery, and can involve a potentially prolonged period of healing and recovery in patients. Similarly, maximal exercise testing can also cause prolonged discomfort to patients and also has the remote danger of leading to an acute episode of rhabdomyolysis and myoglobinuria in the ISCU Myopathy patients.
FGF-21 shows promise as a diagnostic biomarker for mitochondrial diseases, as Suomalainen et al. recently reported the results of a large retrospective clinical study in which FGF-21 levels were investigated in plasma from a broad spectrum of patients affected by mitochondrial diseases. This study reported that FGF-21 was a better prognostic indicator of mitochondrial myopathy than any other plasma biomarker, including lactate and creatine. More importantly, FGF-21 also appeared to correlate with the severity of the mitochondrial myopathy symptoms. We demonstrated that FGF-21 was significantly elevated in ISCU myopathy patients. Thus, FGF-21 could be used to aid in the differential diagnosis of diseases such as ISCU Myopathy, and may help high-risk patients avoid biopsies and exercise tests. Moreover, FGF-21 may serve as a good diagnostic indicator for the efficacy of experimental therapies for treatment of ISCU Myopathy.

In summary, we have identified novel roles for Fe-S clusters in human development and disease. It is our hope that our findings will lead to a better appreciation of the idea that Fe-S clusters lie at the intersection between inorganic biochemistry and human physiology, and that some of this work will lead to the development of new treatments in the clinic.
74. Nordin A, Larsson E, Holmberg M. The defective splicing caused by the ISCU intron mutation in patients with myopathy with lactic acidosis is repressed by PTBP1 but can be derepressed by IGF2BP1. Hum Mutat. 2011.


