Metabolic Catastrophe in Mice Lacking Transferrin Receptor in Muscle

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ABSTRACT

Transferrin receptor (Tfr1) is ubiquitously expressed, but its roles in non-hematopoietic cells are incompletely understood. We used a tissue-specific conditional knockout strategy to ask whether skeletal muscle required Tfr1 for iron uptake. We found that iron assimilation via Tfr1 was critical for skeletal muscle metabolism, and that iron deficiency in muscle led to dramatic changes, not only in muscle, but also in adipose tissue and liver. Inactivation of Tfr1 incapacitated normal energy production in muscle, leading to growth arrest and a muted systemic metabolic derangement.

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1. Introduction

Iron, required by all mammalian cells, circulates bound to plasma transferrin (Tf). Cells express transferrin receptor (Tfr1), which can bind Fe-Tf and internalize it by receptor-mediated endocytosis (Hentze et al., 2004). Vesicles bearing Fe-Tf/Tfr1 fuse with endosomes, where low pH releases iron from Tf for transmembrane transfer to the cytoplasm. Iron is then stored, used directly, or incorporated into heme or Fe–S clusters for utilization in globin proteins, cytochromes, oxidative phosphorylation complexes and other enzymes. Apo-Tf and Tfr1 recycle to the cell surface for further rounds of iron uptake.

Although Tfr1 is widely expressed, it is not universally required for iron assimilation. Through genetic manipulations in mice, we showed that most early embryonic tissues develop normally in the absence of Tfr1, and most adult tissues can form from Tfr1−/− embryonic stem cells (Levy et al., 1999; Ned et al., 2003). Furthermore, most tissues are not iron deficient in mice with a congenital deficiency of Tf, but rather accumulate excess iron (Trenor et al., 2000). However, hematopoietic cells, particularly erythroid precursors, have a critical requirement for Tfr1 (Levy et al., 1999; Ned et al., 2003) and Tf (Trenor et al., 2000). We hypothesized that non-hematopoietic cells might use alternative iron uptake mechanisms, and such mechanisms have been described (Sturrock et al., 1990; Barisani et al., 1995; Baker et al., 1998; Yang et al., 2002; Oudit et al., 2003; Liuzzi et al., 2006; Li et al., 2009). We considered that skeletal muscle is a major site of iron utilization (Andrews, 1999), and asked whether Tfr1 was important.

We found that skeletal muscle has a critical requirement for Tfr1. Mice in which the muscle Tfr1 gene has been inactivated during embryogenesis appeared normal at birth, but rapidly developed a dramatic and lethal phenotype. They stopped growing, lost adipose tissue, developed hepatic steatosis and experienced profound hypoglycemia before death. Although Tfr1 was intact in hepatocytes, the liver also became iron deficient early in the development of the phenotype and decreased production of the iron regulatory hormone hepcidin. The abnormal phenotype was corrected by administration of parenteral iron to saturate transferrin, increasing non-Tf-bound iron, indicating that neonatal muscle iron deficiency was responsible for the severe, systemic metabolic derangement.

2. Materials and Methods

2.1. Animal Experiments

We crossed 129/SvEv mice bearing a floxed Tfr1 (gene symbol Tfrc) allele (Chen et al., 2015) with B6.Cg-Tg (ACTA1-cre)79Jme/J mice...
(Jackson Laboratory #006,149) expressing Cre recombinase under the control of the human skeletal muscle actin promoter (Miniou et al., 1999). We backcrossed with 129/SvEv mice for more than 10 generations. As indicated in the figure legends, microarray experiments and experiments shown in Fig. 1A,C,F,G,H,I, and Fig S1C used mice with similar, mixed 129/C57BL6 backgrounds and experiments shown in all other figures used mice with a homogeneous 129/SvEv background. Apart from several days' difference in length of survival, the phenotypes were very similar on both backgrounds. Animals were genotyped by PCR using genomic DNA from toe clips (Truett et al., 2000). Primers are described in Table S1. Blood glucose was measured at P6 using the FreeStyle Freedom Lite monitoring system (Abbott). Insulin levels were measured using a Rat/Mouse Insulin ELISA kit (EMD Millipore) according to the manufacturer’s protocol. Total ketones were measured using a Total Ketone Bodies kit (Wako Diagnostics) according to the manufacturer's protocol. Glycogen measurements were performed as previously described (Thyfault et al., 2007). Non-heme iron was measured as previously described (Levy et al., 1999). For iron rescue experiments, mice were injected IP with 5 mg of Uniferon® 100 (25 μg) at P3, or at both P3 and P21. All procedures were carried out under protocols approved by the Duke Animal Care and Use Committee. Sample sizes included in animal protocols were determined with assistance from the Duke Biostatistics Core.

2.2. RNA Extraction, Microarray Analysis and Quantitative PCR

RNA was extracted using an RNA isolation kit (Qiagen) according to the manufacturer’s instructions. Samples were treated with DNase I (Qiagen) to remove genomic DNA. Microarray analysis was performed using Affymetrix Mouse Expression Array 430A 2.0. RNA quality control, hybridization and data analysis were performed by the Duke University Microarray Shared Resource (see Supplemental Experimental Procedures) and results have been deposited in the NCBI Gene Expression Omnibus database, accession number GSE68675. For quantitative PCR, cDNA from liver and skeletal muscle was synthesized using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Real time quantification was performed using iQ SYBR Green Super mix (Bio-Rad). The primer sequences are described in Table S1.

2.3. Immunoblots

Protein lysates were prepared from muscle and liver using RIPA buffer (50 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1% NP40, 0.1% SDS, 0.5% Na deoxycholate), protease inhibitors and phosphatase inhibitors (Roche Applied Science). Quantification was performed using Bio-Rad DC Protein Assay (Bio-Rad) and transferred to Immune-Blot® PVDF membrane (Bio-Rad). Membranes were incubated with primary and secondary antibodies listed in Table S2, developed with chemiluminescence ECL Western blotting detection solutions (GE Healthcare) and exposed to autoradiography film (HiBlot CL®, Denville Scientific). Signal intensity was quantified using Image J software.

2.4. Morphometric analysis

Tibialis anterior (TA) muscle was isolated from mice and immediately flash frozen in embedding media. Muscle cross-sections were stained with Alexa Fluor® 594 WGA (Life Technologies, Invitrogen) and individual fibers were counted using Image J software.

2.5. Oil Red O Staining

Liver was harvested from mice and frozen in embedding media. 10 μm sections were stained with Oil Red O as previously reported (Carson and Hladlik, 2009).

2.6. IRP-IRE Binding Assay

IRP-IRE binding assays were carried out using the LightShift™ Chemiluminescent RNA EMSA according to manufacturer’s instructions (Pierce Biotechnologies). The IRE sequence is shown in Table S1.

2.7. Enzymatic Assays

Aconitase activity was measured in isolated mitochondria using the Aconitase Enzyme Activity Microplate Assay Kit (Abcam) according to the manufacturer’s instructions. Mitochondria were isolated as previously described (Wieckowski et al., 2009). Complex II activity was measured in gastrocnemius tissue lysates as previously reported (Spinazzi et al., 2011).

2.8. Metabolomics Analyses

Amino acids, acylcarnitines and organic acids were analyzed using stable isotope dilution techniques. Amino acids and acylcarnitine measurements were made by flow injection tandem mass spectrometry as described previously (An et al., 2004; Wu et al., 2004). The data were acquired using a Waters Acquity™ UPLC system equipped with a triple quadrupole detector and a data system controlled by MassLynx 4.1 operating system (Waters). Organic acids were quantified using methods described previously (Jensen et al., 2006) employing Trace Ultra GC coupled to ISQ MS operating under Xcalibur 2.2 (Thermo Fisher Scientific).

2.9. Proteomics Analyses

Proteomics analysis on 6 gastrocnemius samples was carried out by the Duke Proteomics and Metabolomics Shared Resource (see Supplemental Experimental Procedures). Proteomics results have been deposited in the MassIVE database (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp), ID MSV000079328.

2.10. Statistical Analyses

Unpaired Student’s T tests were performed using Microsoft ® Excel® 2011 software. P values less than 0.05 were considered significant. Representation of the p values are as follows: not significant (ns), p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.****p ≤ 0.0001.

3. Results

3.1. Tfr1 Deficiency in Skeletal Muscle Causes early Postnatal Death

We selectively inactivated the murine Tfr1 gene using a skeletal muscle actin (HSA)-Cre transgene, expressed from embryonic day 9 and highly specific for skeletal muscle (Miniou et al., 1999), to recombine loxP sites flanking Tfr1 exons 3 to 6 (Chen et al., 2015) to generate Tfr1mut/mut mice. Tfr1mut/mut mice were born in Mendelian ratios. We confirmed that they expressed little Tfr1 mRNA or protein in skeletal muscle (Fig S1). They were slightly smaller than Tfr1fl/fl wild type (WT) littermates at birth, fell behind in growth (Fig 1A,B) and typically died or had to be euthanized before P13 on a mixed background or P11 on a homogeneous 129/SvEv background. Because the males usually lived longer and therefore had larger tissues, we continued our analysis with male mice unless otherwise noted. Necropsy revealed that the Tfr1mut/mut mice had small muscles (Fig 1C,D) but the total number
of fibers was preserved (Fig. 1E). We did not observe degenerative changes such as central nuclei or misalignment of Z-lines but we saw lipid droplets in diaphragmatic muscle by electron microscopy (not shown).

We found striking changes in other tissues, even though Tfr1 was targeted only in muscle. Fat pads were present early in life in Tfr1\textsuperscript{mu/mu} mice (not shown) but disappeared by the time animals showed distress (Fig. 1F). Before growth delay was apparent the abdominal organs appeared grossly normal (not shown), but later the Tfr1\textsuperscript{mu/mu} liver contained excess neutral lipid and both liver and spleen were small compared to controls (Fig. 1G–I). Although normal at day 4 (not shown), serum glucose became low as the phenotype developed (Fig. 1J). At P6 serum insulin ranged from 0.5 to 6.7 ng/mL in control animals (n = 18) but was undetectable in almost all mutants (n = 17). Two mutant animals that did have measurable serum insulin (1.3 and 1.5 ng/mL) also had normal serum glucose (117 and 94 mg/dL respectively), suggesting that they had not yet developed the full phenotype. Low serum glucose, coupled with non-detectable serum insulin, would exacerbate the glucose deficit in insulin-responsive tissues – muscle and fat – likely contributing to the phenotype. Serum ketones were elevated at P6 in both control and mutant animals, consistent with their young age, but markedly more elevated in the mutants (Fig. 1K). Surprisingly, liver glycogen was indistinguishable between controls and mutants, but muscle glycogen was decreased in Tfr1\textsuperscript{mu/mu} mice (Fig. S1D). The animals may have succumbed to hypoglycemia, but attempts to rescue the animals by administering intraperitoneal glucose did not substantially prolong survival (not shown). Alternatively, they may have died from respiratory failure due to diaphragmatic insufficiency. We speculate that normal placental function spared the mother (Wang et al., 1995) and that changes developed when the animals no longer benefited from the placenta’s capacity to correct metabolic abnormalities and substrates for gluconeogenesis decreased, as described later.

3.2. Muscle Iron Deficiency and Impaired Oxidative Phosphorylation

We asked whether skeletal muscle was iron deficient in the absence of Tfr1. In mice with homogeneous 129 backgrounds and earlier onset of the phenotype, the total amount of muscle iron, per gram of tissue, was similar between Tfr1\textsuperscript{mu/mu} mice and controls at P6 as measured by inducively coupled plasma mass spectrometry (not shown). However, muscle ferritin was decreased (Fig. 2A,B) and iron regulatory protein binding activity was increased in Tfr1\textsuperscript{mu/mu} animals at P6 (Fig. 2C,D), indicative of iron deficiency. Myoglobin was decreased, consistent with insufficiency of its co-factor heme (Fig S2A,B). This suggests that the animals had a normal endowment of iron at birth, but additional iron was not assimilated for muscle growth and functional iron deficiency ensued.

Heme and Fe–S clusters are critical cofactors for electron transport chain (ETC) complexes and failure of mitochondrial respiration might contribute to the phenotype observed in Tfr1\textsuperscript{mu/mu} mice. We evaluated the integrity of ETC complexes I to V at P6 by immunoblotting with a cocktail of antibodies that detects labile protein components of each. Although complex V was represented similarly in WT and Tfr1\textsuperscript{mu/mu} mice, the mutants were deficient in the other complexes (Fig. 2E,F), which require iron (Xu et al., 2013). We confirmed complex II deficiency by enzymatic assay (Fig. 2G) and also observed that a key Fe–S containing protein of complex I, Ndufs3, was markedly decreased (Fig. 2H). The deficiencies in ETC complexes did not appear to result from a substantial decrease in the number of mitochondria because complex V levels (Fig. 2E) were similar in WT and Tfr1\textsuperscript{mu/mu} mice and expression of mitochondria-encoded mRNA transcripts (Fig S2C) was not substantially different between WT and Tfr1\textsuperscript{mu/mu} mice. These results support the interpretation that Tfr1\textsuperscript{mu/mu} muscle was iron deficient, leading to impaired mitochondrial respiration.

3.3. Tfr1\textsuperscript{mu/mu} Mice are Rescued by Iron

We hypothesized that if Tfr1 is needed for iron assimilation by skeletal muscle, it might be possible to rescue the mutant phenotype by causing iron overload. We knew that muscle could take up non-Tf-bound iron in animals with a congenital deficiency of Tf (Trenor et al., 2000). We treated WT and Tfr1\textsuperscript{mu/mu} mice with a large dose of iron dextran at P3 to increase circulating non-Tf-bound iron. With this treatment, Tfr1\textsuperscript{mu/mu} mice appeared healthy, had normal body weights (Fig. 3A,B), survived for at least 4 weeks with no further intervention, and survived for substantially longer with a second treatment. Both WT and Tfr1\textsuperscript{mu/mu} mice had marked tissue iron overload as expected, and the extent of iron loading was similar (Fig. 3C,D; note log scale). H-ferritin was similarly increased in mice of both genotypes (Fig. 3E,F). At 4 weeks Tfr1\textsuperscript{mu/mu} mice had normal serum glucose levels (Fig. 3G). We conclude that iron deficiency is the root cause of the mutant phenotype, and that it can be rescued by administration of excess iron.

3.4. Decreased Liver Iron in Tfr1\textsuperscript{mu/mu} Mice

Hepatic steatosis and hypoglycemia indicated that muscle iron deficiency led to changes in the liver, even though the Tfr1 gene was intact and expressed (Fig. 4A). While we observed substantial variability, likely due to variation in the onset of the abnormal phenotype, liver non-heme iron was unexpectedly decreased at P6, and H-ferritin was reduced, indicating liver iron deficiency (Fig. 4B–D). Consistent with hepatic iron deficiency, activity of the Fe–S cluster-dependent enzyme mitochondrial aconitase was decreased (Fig. 4E) and mRNA encoding the iron regulatory hormone hepcidin was markedly diminished (Fig. 4F). Decreased liver iron did not appear to be due to systemic iron deficiency because hemoglobin and hematocrits were not significantly different between P6 mutant and control mice (not shown). These results suggest that loss of Tfr1 in skeletal muscle led to hepatic iron deficiency.

3.5. Gene Expression Changes in Tfr1\textsuperscript{mu/mu} Muscle and Liver

We profiled mRNA expression changes in Tfr1\textsuperscript{mu/mu} tissues relative to WT controls [results deposited online (Barrientos and Andrews, 2015)]. In this experiment we used mice on a mixed background because they lived longer and therefore had larger tissues for mRNA extraction. We analyzed muscle and liver from mice at P5 (early), before the phenotype was grossly apparent, and P9 (late), when the phenotype had developed and the animals were within several days of death. We used Gene Set Enrichment Analysis [GSEA; (Subramanian et al., 2005; Mootha et al., 2003)] to look for informative patterns. Few genes were differentially regulated in Tfr1\textsuperscript{mu/mu} muscle early, but Tfr1 mRNA containing non-deleted exons was upregulated, consistent with iron deficiency. Other upregulated genes included Ccna2d1, a subunit of a voltage-dependent calcium channel that has been implicated in non-Tf-bound iron uptake in the heart, (Oudit et al., 2003), angiopoietin-like 4 (Angptl4), carbonic anhydrase 2 (Car2) and retinol saturase (Retrsat). The most strongly downregulated genes in early muscle included frizzled b (Fzb2), low density lipoprotein receptor-related 2 binding protein (Lrp2bp), H-ras-like suppressor (Hrasl5), growth factor receptor-bound protein 14 (Grb14), contactin associated protein-like 2 (Cntnap2) and myosin heavy chain 4 (Myh4).

Later, after the phenotype became apparent, more genes were differentially regulated in mutant muscle. Upregulated genes were
annotated by GSEA as associated with hypoxia,1 Mapk signaling,2 glycolysis3, Ppar signaling,4 fatty acid metabolism5 and Tnfx signaling.6 Predicted targets of Nfα2 and estrogen receptor-related protein α (Esra, also called Nr3B1)6 were particularly prominent. The most strongly upregulated gene was ankyrin repeat domain 2 (Ankrd2), which has been reported to inhibit myoblast differentiation (Mohamed et al., 2013). Angpt4 and brain-derived neurotrophic factor (Bdnf), both induced by fasting (Kersten et al., 2009; Walsh et al., 2015), were also upregulated. Genes downregulated in late muscle were annotated as being involved in metabolism of amino acids, heme metabolism7 and mTorc1 signaling.10

We also examined hepatic gene expression. We saw many changes early, before steatosis was apparent. The most prominent categories of upregulated genes were those involved in Tnfα signaling via NF-κB,11 apoptosis12, hypoxia13, lipid and lipoprotein metabolism,14 insulin signaling,15 PPAR signaling16 and fatty acid, triacylglycerol and ketone metabolism.17 The most strongly upregulated were regulator of G-protein (G) protein (Rgs16), leptin receptor (Lepr), oxidative stress growth inhibitor 1 (Osg1t1), D site albumin promoter binding protein (Dbp) and pantothenate kinase 1 (Ppap2b) genes. Genes downregulated in early liver included, strikingly, more than 50 genes, mostly E2F targets, which are involved in DNA replication and the cell cycle. The most strongly downregulated gene was Hump, encoding the iron regulatory hormone hepcidin, consistent with Fig. 4F.

After the phenotype became apparent, at the late time point, more genes were differentially regulated in the mutant liver. Upregulated genes were annotated in GSEA as involved in Tnfα signaling, metabolism of lipids and lipoproteins, insulin signaling, adipocytokine signaling, carbohydrate metabolism and lipid, fatty acid, triacylglycerol and ketone body metabolism. Rgs16, Lepr and Pank1 were again among the most highly induced, along with G protein–coupled receptor 64 (Gpr64), cytochrome P450 family 2 subfamily b, polypeptide 39 (Cyp2b39), adiponectin A-V (Apop4) and thiorodoxin interacting protein (Txnip). Transcripts encoding enzymes involved in gluconeogenesis – Ldhb, encoding a subunit of lactate dehydrogenase, Pck1, encoding phosphoenolpyruvate carboxylase, and G6pc, encoding glucose-6-phosphatase – were markedly increased, as were hepatocyte nuclear factor 4 alpha (Hnf4α) and nuclear receptor subfamily 4, group A, member 1 (Nr4a1), both transcription factors that induce gluconeogenesis. Of note, Hnf4α has been reported to repress hepcidin expression (Courselaud et al., 2002). Genes downregulated in the mutant liver at the later time included many E2F target genes involved in the cell cycle, transmembrane receptors, and genes involved in heme metabolism. The most strongly downregulated genes were Slc22a7, Slc22a29, both organic anion transporters. Other members of the Slc22 family of organic anion transporters were also downregulated.

3.6. Metabolic Changes

To better understand the complex phenotype of Tfr1mu/mu mice, we considered molecular events that might cause arrested growth, hepatic steatosis and hypoglycemia. We examined skeletal muscle after the phenotype was grossly apparent and observed increased expression of Bdnf mRNA (microarray data) and increased phosphorylation of AMPK (Fig 3A), consistent with an attempt to stimulate fatty acid oxidation and glucose uptake. We also observed decreased phosphorylation of AKT, consistent with a decrease in muscle growth, and, accordingly, decreased phosphorylation of proteins downstream of the AKT target mTOR (Fig S3B–D). The ratio of Mapl1c3–II (LC3-II) to LC3-I was increased, suggesting induction of autophagy (Fig S3E). All of these changes represent responses to energy insufficiency or nutrient deprivation.

To evaluate metabolic changes, we carried out a metabolomics analysis on muscle, liver and serum from WT and Tfr1mu/mu mice at P4 and P6 on a 129 background, before and after the phenotype was apparent, respectively (Fig. 5). All results are shown because there were qualitative and quantitative differences between the two time points, but for simplicity the P6 results, from after the phenotype had developed, will be discussed primarily.

In Tfr1mu/mu mice we observed increased accumulation of three tricarboxylic acid (TCA) cycle intermediates – citrate, α-ketoglutarate, and succinate, as well as succinyl-carnitine (C4-DC), which derives from succinyl-CoA – and decreased amounts of fumarate and malate. These changes may be attributable to decreased activity of aconitase and succinate dehydrogenase enzymes, which both require Fe–S cluster co-factors. We also saw progressive elevation of C4OH (3-OH butyryl carnitine) suggesting increasing ketone delivery to or ketosis within the mutant muscle. Odd chain acylcarnitines (C5OH, C5:1, C3DC) were increased, consistent with increased amino acid catabolism and diminished amino acids detected in muscle. Increased branched chain amino acids (leucine, isoleucine) probably contributed to the increase in C5OH. Some β oxidation intermediates were slightly increased, but interestingly not to levels typically observed during starvation or exercise. Tfr1mu/mu muscle showed diminished anaplerotic amino acids, consistent with increased amino acid catabolism in an attempt to fuel the TCA cycle. Aspartic acid, one of the anaplerotic amino acids, is also used for several transamination reactions. Importantly, there were decreased levels of lactate and alanine, both precursors for gluconeogenesis that are typically shuttled from muscle to liver during starvation and exercise.

We observed increased muscle citrate, usually a signal of plenty, which can inhibit phosphofructokinase and glycolysis and may inhibit β oxidation indirectly. At the same time, impaired oxidative phosphorylation may lead to an increased AMP/ATP ratio and activation of AMPK (Fig 3A), which is a signal of energy insufficiency. In addition to these mixed signals, we observed increased acylcarnitine (C2), which derives from acetyl CoA and might reflect mitochondrial accumulation of this crucial metabolite.

The livers of Tfr1mu/mu mice should stabilize serum glucose through gluconeogenesis but the mice became hypoglycemic towards the end of the first week of life. A previous report linked hepatocyte iron deficiency to decreased gluconeogenesis in an in vitro system (Klemppa et al., 1989) but in our animals changes in the metabolomics profile suggested that amino acid insufficiency was the reason for failure of gluconeogenesis. Amounts of almost all amino acids were severely decreased in serum at P6. Hepatic citrate was markedly elevated, in spite of metabolic stress, possibly because diminished liver iron caused decreased mitochondrial acetyl coenzyme a activity (Fig. 4E). In contrast to muscle, where beta-oxidation may have been impaired due to ETC deficiencies, there was a strong signature for hepatic fatty acid catabolism, with markedly elevated even chain acylcarnitines.

Serum from Tfr1mu/mu mice was also more severely affected at P6 than P4. The older mice had increased C4OH suggesting increased ketone delivery or ketolysis in tissues such as muscle and heart. There
was a robust increase in several long chain acylcarnitines derived from long chain acyl-CoA intermediates of β oxidation. As noted earlier, all amino acids were strongly diminished, likely contributing to the failure of gluconeogenesis.

3.7. Proteomic Analysis

To further evaluate the phenotype, we subjected muscle proteins from P6 (late) WT and Tfr1mut/mut mice to mass spectrometry-based proteomic analysis. We assessed relative protein expression levels and lysine acetylation modifications (data online at https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp, ID MSV000079328). The relative expression levels of most proteins were not different between WT and mutant muscle samples. However, consistent with mRNA profiling results, the amounts of myosin heavy chain 4 (Myh4) and eukaryotic translation elongation factor 1 alpha 2 (Eef1a2) were strongly diminished and the amount of acetyl-coA acyltransferase 2 (Acaa2) was increased (Fig. 6A). The most striking changes were in proteins that...
did not show altered mRNA expression: components of ETC complexes I (Ndufa5, 6, 9, 10; Ndufb9; Ndufs1, 3, 4, 5, 7, and Ndufv1), II (Sdha, Sdhb), and cytochrome C were all depleted in mutant mice compared to WT animals (Fig. 6A). Each of these complexes and many of these proteins use Fe–S clusters or heme as co-factors. Others have reported decreased expression of ETC proteins in iron deficient muscle (Rensvold et al., 2013) and noted that mRNA and protein levels did not always correlate. Decreased ETC proteins may reflect instability of their complexes when iron is insufficient.

We examined the acetyl-proteome of P6 Tfr1mut/mut muscle by isolating lysine-acetylated peptides using an anti-acetyl lysine antibody followed by mass spectrometry analysis (Fig. 6B, Table S3). Strikingly, nearly all proteins with increased acetylation in the mutant were mitochondrial. Multiple subunits of complex V, which do not contain iron and were not diminished in total protein levels, were hyperacetylated. Furthermore, nearly every enzyme of the TCA cycle was multiply hyperacetylated. The only exception was succinate dehydrogenase, an iron-containing enzyme shared with oxidative phosphorylation. As...
mentioned earlier, amounts of Sdha and Sdhb were diminished, likely resulting in decreased activity of that enzyme.

Mitochondrial aconitase, Aco2, was the most highly hyperacetylated protein in Tfr1 mu/mu muscle (Table S3). While it was hyperacetylated on multiple residues, acetylation on K245 was markedly increased relative to control muscle. K245 would be buried within the protein and inaccessible in its native conformation (Dick Brennan, Duke Biochemistry, personal communication). Furthermore, acetylation of K245, if it occurred before protein folding, would disrupt the normal conformation. As mentioned earlier, Aco2 contains an Fe–S cluster, which may be limiting in iron-deficient cells. We speculate that, similar to what has been shown with Aco1 (Walden et al., 2006), unavailability of the Fe–S cluster may result in a dramatic conformational change in Aco2, making K245 accessible for acetylation. This would be consistent with the increase in muscle citrate we observed (Fig. 5).

Acetylation of TCA cycle enzymes has been reported (Anderson and Hirschey, 2012) but its consequences are not well understood. If...
acetylation activates the enzymes, it might represent an attempt to increase the activity of the TCA cycle. However, in the absence of a functional ETC, this would be ineffective. Furthermore, it appears that the pyruvate dehydrogenase complex may be inactive, causing a switch away from the TCA cycle by inhibiting conversion of pyruvate to acetyl CoA. We observed increased acetylation of K321 of pyruvate dehydrogenase alpha 1 (Pdha1) (Table S3), which decreases its activity (Ozden et al., 2014), and increased phosphorylation of S293 (Fig S3F), which has the same effect (Rardin et al., 2009). The phosphorylation of S293 may be carried out by pyruvate dehydrogenase kinase isoenzyme 4 (Pdk4) and Pdk4 mRNA was markedly increased in our microarray profiles.

Interestingly, many enzymes important for β oxidation were also hyperacetylated (Fig. 6B, Table S3). Acetylation of these proteins has been described in normal heart (Foster et al., 2013) and other cell types (Kim et al., 2006) but for the most part the functional consequences have not been determined. Lysine acetylation of long chain acyl-CoA dehydrogenase (Acadl) on two specific residues was shown to inhibit activity (Bharathi et al., 2013), but neither of those residues was hyperacetylated in our mutant muscle samples.

Increased mitochondrial acetyl CoA might drive enzymatic or non-enzymatic acetylation of mitochondrial proteins. Furthermore, decreased NAD levels, predicted due to impairment of complex I activity, could contribute to hyperacetylation by inhibiting sirtuin deacetylase activity. We noted that many of the acetylation events we observed corresponded to previously described targets of sirtuin 3 (Sirt3), a mitochondrial deacetylase (Rardin et al., 2013). Although we did not attempt to measure Sirt3 activity directly, it is likely impaired, contributing to the metabolic defects. We compared our results to results from Sirt3−/− muscle proteomic analysis (CR Kahn, B Gibson, M Rardin personal communication of unpublished results) and found that, of a total of 349 hyperacetylated peptides in Tfr1 mu/mu muscle and 397 hyperacetylated peptides in Sirt3−/− muscle, there were 70 peptides common to both datasets that changed in the same direction (Table S4).
4. Discussion

We found that Tfr1 is important in murine skeletal muscle, and that iron deficiency consequent to the loss of Tfr1 set off a chain of events that resulted in systemic metabolic abnormalities and early postnatal lethality. Tfr1^{mu/mu} mice appeared normal at birth and had normal numbers of skeletal muscle fibers, making it unlikely that they suffered a developmental defect. Furthermore, there was no evidence of muscle atrophy or necrosis. However, over the first week of life the mutant animals stopped growing, lost body fat, developed hepatic steatosis and became incapable of maintaining normal serum glucose levels, with death ensuing before the end of the second week. This dramatic

Fig. 5. Metabolic changes in Tfr1^{mu/mu} mice. (Left panel) Heat map of metabolomics results from gastrocnemius muscle, liver and serum from P4 (4d) and P6 (6d) WT and Tfr1^{mu/mu} mice (males; 129 background). Blue — decreased, red — increased, white — not significantly different from WT, gray — not determined; SC — short chain, MC — medium chain, LC — long chain, VLC — very long chain, AC — acylcarnitine. Because the animals were small, we pooled tissue from 3 to 4 mice for each sample in this analysis. At P4 we analyzed 6 pools each for WT and Tfr1^{mu/mu} (total 23 mice of each genotype). At P6 we analyzed 6 pools each for WT and Tfr1^{mu/mu} (total 18 mice of each genotype). P4 Tfr1^{mu/mu} mice were compared to P4 WT mice and P6 Tfr1^{mu/mu} mice were compared to P6 WT mice. (Right panels) Diagrammatic representation of pathways changing in the muscle (top) and liver (bottom) of Tfr1^{mu/mu} mice. Metabolites highlighted in blue are decreased and metabolites highlighted in red are increased.
phenotype was fully reversed by administration of high dose iron dextran, showing that muscle iron deficiency was the root cause.

Ineffective iron assimilation, due to loss of Tfr1, disrupted muscle metabolism. Representative proteins from iron-containing ETC complexes I to IV were decreased and enzymatic assay confirmed that complex II activity was impaired in skeletal muscle when the phenotype was apparent. Complex V subunits and TCA cycle enzymes were hyperacetylated. While the consequences of hyperacetylation are uncertain for most of these enzymes, in one case we can infer a functional outcome. Aco2, the mitochondrial aconitase involved in the TCA cycle, was highly acetylated on a lysine residue that is not normally exposed. Its accessibility indicates that Aco2 was acetylated when it was not properly folded, likely because of unavailability of Fe–S clusters due to iron deficiency, consistent with the decreased Aco2 activity and accumulation of citrate in muscle. Increased Pdk4 expression and specific acetylation and phosphorylation events affecting Pdha1 likely

Fig. 6. Proteomic analysis shows increased acetylation in proteins in muscle of Tfr1<sup>mu/mu</sup> mice. (A) Heat map showing the global protein expression of three pools of Tfr1<sup>mu/mu</sup> gastrocnemius (9–10 mice per pool) and three pools of WT gastrocnemius (7 mice per pool) at P6 (males; 129 background). The table lists proteins with significant changes in expression in Tfr1<sup>mu/mu</sup> mice compared to WT mice. (B) Heat map of acetyl proteome of three Tfr1<sup>mu/mu</sup> and three WT mice at 6 days of age. We used the same pooled samples as in (A). The table lists proteins with significant increase in acetylation in Tfr1<sup>mu/mu</sup> mice compared to WT mice.
inhibited the pyruvate dehydrogenase complex in skeletal muscle, precluding conversion of pyruvate to acetyl CoA. This may have caused a switch to attempted use of β-oxidation of fatty acids and/or ketones in muscle. However, we can only infer the metabolic consequences, because we have not done comprehensive metabolic flux experiments, which would be very difficult in newborn mice.

Metabolic changes were not limited to skeletal muscle, although Tfr1 was only deficient in that tissue. Body fat pads disappeared, suggesting that lipolysis ensued, presumably to provide a source of energy for muscle, and the liver became steatotic. Tfr1-null muscle showed increased expression of Angptl4 mRNA, suggesting a starvation-like response. Starvation should promote lipolysis for increased fatty acid delivery to the muscle, but failure of the ETC would preclude effective β oxidation. We do not know why the levels of acylcarnitines were lower in muscle than liver, but this might suggest a defect in muscle fatty acid uptake or in the activity of carnitine palmitoyltransferase 1 (Cpt1). The presence of lipid droplets in the diaphragm was consistent with increased fatty acid delivery and decreased β oxidation. Angptl4 expression has also been associated with hypoglycemia, inhibition of gluconeogenesis and hepatic steatosis (Xu et al., 2005), as observed in our mice.

There was evidence that the liver was attempting to respond by increasing gluconeogenesis to provide glucose for muscle metabolism. Several mRNAs that encode proteins inducing gluconeogenesis were increased in the liver, including G6pase (Pashkov et al., 2011), Pank1 (Leonardi et al., 2010) and Txnip (Muñoz, 2007). Lactate dehydrogenase b (Ldhb) was markedly induced, suggesting that the liver was trying to use lactate to produce pyruvate through the Cori cycle. Transcripts encoding phosphoenolpyruvate carboxykinase (Pck1) and the catalytic subunit of glucose-6-phosphatase (G6pc) were also increased. We note that expression of Pck1 and G6pc is controlled by Rev.-erbα (Nri1d1), a transcription factor that binds heme, and heme binding is reported to repress Pck1 and G6pc expression (Yin et al., 2007). We speculate that decreased availability of heme, either from iron deficiency in skeletal muscle or liver, de-repressed expression of these gluconeogenic enzymes. However, in spite of their increased expression, Tfr1-null mice developed progressive hypoglycemia, indicating that gluconeogenesis failed. This is likely attributable to lack of substrate, as amino acids were profoundly decreased in muscle, serum and liver.

Even before steatosis developed, the liver showed profound downregulation of a large number of genes with various roles in the cell cycle, most of which are annotated as E2f targets. We are not certain why we see this striking pattern, but we note that Txnip, which is highly expressed in mutant muscle and liver, has been shown to cause cell cycle arrest (Han et al., 2003).

Severe iron deficiency in skeletal muscle compromises production of Fe–S clusters important for ETC complexes and other enzymes. However, our Tfr1-null muscle differed from muscle from human patients with Fe–S cluster depletion due to deficiency of the Fe–S cluster scaffold protein ISCU (Crooks et al., 2014). Similar to our mice, those patients had decreased mitochondrial aconitase activity, as expected. There were some similarities in gene expression but they were modest, and muscle mRNA expression of several key genes, including Tsf, AlaS1 and SLC25A28 was increased in the human patients, while expression of those genes or close homologs (Tst, AlaS2, SLC25A37) was strongly decreased in muscle in our mice. Crooks et al. observed that fibroblast growth factor 21 (FGF21) mRNA was markedly induced in muscle samples from affected patients, but Fgf21 was not induced in muscle from our mice (not shown). Furthermore, the patients were not reported to have hepatic steatosis or hypoglycemia. These differences suggest that loss of Tfr1 in skeletal muscle has consequences beyond depletion of Fe–S clusters and the proteins that require them.

The pattern of increased lysine acetylation we observed by proteomic analysis of Tfr1-null skeletal muscle was striking in two ways. First, nearly all hyperacetylated proteins were mitochondrial and most hypoacetylated proteins were not. Second, there was strong concordance between acetylation events we observed and those observed in Sirt3−/− muscle. Both observations suggest that Sirt3 may be less active in Tfr1-null muscle, though we cannot exclude the possibility that increased mitochondrial acetyl CoA resulting from decreased mitochondrial aconitase activity, or increased acetylase activity, contributed to this pattern. It is possible that mitochondrial dysfunction has led to a deficit in acetyl donors outside of the mitochondria.

Surprisingly, iron deficiency in skeletal muscle also led to iron deficiency in the liver before the systemic phenotype was apparent. This seems counterintuitive—one might expect that a decrease in the ability of the muscle to extract iron from circulating Tf might lead to increased hepatic iron stores, but that was not what we observed. There are several possible explanations. First, muscle iron deficiency might instigate an as yet unidentified signal to the liver to decrease expression of the iron regulatory hormone hepcidin and mobilize hepatic iron stores. A similar signal has been described from the erythroid bone marrow to regulate intestinal iron absorption (Finch, 1994), apparently by decreasing hepcidin expression (Hentze et al., 2004). It was recently reported that erythropherrone (Fam132b) might be a component of this “erythroid regulator” (Kautz et al., 2014). Expression of erythropherrone was not increased in Tfr1null/mu muscle (not shown), even though this protein has also been identified as myonectin and described as linking muscle activity to adipose tissue and liver lipid metabolism (Seldin et al., 2012). Alternatively, metabolic changes associated with iron deficiency in Tfr1null/mu muscle might alter hepatic iron homeostasis in a manner not yet described. Regardless of the mechanism, decreased expression of the iron regulatory hormone hepcidin should result in increased iron availability to skeletal muscle. However, our results indicate that this is futile when Tfr1 is inactive in muscle.

Our experiments have allowed us to examine the local and systemic sequelae of severe, isolated muscle iron deficiency. While impairment of iron-dependent enzymes in muscle might have been predicted, the systemic effects are profound and surprising, and elucidate a previously unappreciated link between iron homeostasis and intermediary metabolism. We have studied an extreme situation, contrived by targeted disruption of Tfr1, but we speculate that less severe muscle iron deficiency may have hitherto unappreciated effects on systemic energy homeostasis. This could be quite significant clinically, as iron deficiency affects at least 15% of the world’s population and is the third most prevalent cause of years lived with disability (Vos et al., 2012).

**Author Contributions**

TB and NCA designed the experiments and wrote the manuscript. TB carried out most of the experiments with the assistance from IL and MB. EJS and MAM carried out proteomic experiments and analyses. TRK and DMM performed metabolomic analyses and experiments.

**Disclosures**

NCA is a member of the Board of Directors of Novartis AG, but Novartis had no connection to the work described in this paper. There are no conflicts of interest to report.

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Appendix A. Supplementary data

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