

# Fibroblast growth factor 21 drives dynamics of local and systemic stress responses in mitochondrial myopathy with mtDNA deletions

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## Summary

Mitochondrial dysfunction elicits stress responses that safeguard cellular homeostasis against metabolic insults. Mitochondrial integrated stress response (ISR<sup>mt</sup>) is a major response to mitochondrial (mt)DNA expression stress (mtDNA maintenance, translation defects), but knowledge of dynamics or interdependence of components is lacking. We report that in mitochondrial myopathy ISR<sup>mt</sup> progresses in temporal stages, and development from early to chronic is regulated by autocrine and endocrine effects of FGF21, a metabolic hormone with pleiotropic effects. Initial disease signs induce transcriptional ISR<sup>mt</sup> (ATF5, mitochondrial one-carbon cycle, FGF21, GDF15). The local progression to 2<sup>nd</sup> metabolic ISR<sup>mt</sup>-stage (ATF3, ATF4, glucose uptake, serine biosynthesis, transsulfuration) is FGF21-dependent. Mitochondrial unfolded protein response marks the 3<sup>rd</sup> ISR<sup>mt</sup>-stage of failing tissue. Systemically, FGF21 drives weight-loss, glucose-preference and modifies metabolism and respiratory chain deficiency in a specific hippocampal brain region. Our evidence indicates that FGF21 is a local and systemic messenger of mtDNA stress in mice and humans with mitochondrial disease.

## Introduction

Mitochondrial diseases show an unprecedented clinical variability, but molecular mechanisms underlying their tissue-specific manifestations are poorly understood (Gorman et al., 2016; Nunnari and Suomalainen, 2012). Recent evidence suggests that metabolic responses elicited by different mitochondrial insults are specific for tissue type and dysfunction (Suomalainen and Battersby, 2018) raising an exciting question whether the responses contribute to tissue-specific manifestations of disease.

The first mitochondrial stress response, mitochondrial unfolded protein response (UPR<sup>mt</sup>), was characterized in mammalian cultured cells and in the worm *C. elegans* (Durieux et al., 2011; Haynes et al., 2013; Haynes et al., 2007; Merkwirth et al., 2016). Proteotoxic stress activates UPR<sup>mt</sup> through ATFS-1 (stress-activated transcription factor S1) that relocates from mitochondria to nucleus, binds to promoters with amino acid response elements (AAREs), and induces transcription of mitochondrial heat shock proteins (HSPs), chaperones and proteases (Haynes et al., 2013; Nargund et al., 2012). In the worm, neuronal inactivation of mitochondrial cytochrome-c-oxidase elicited UPR<sup>mt</sup> locally, but also in the gut, pointing to a secreted factor mediating stress signals from the affected neurons to periphery (Durieux et al., 2011). The worm “mitokine” was recently reported to be a Wnt-ligand/EGL-20 (Zhang et al., 2018). However, in mice with mitochondrial dysfunction UPR<sup>mt</sup>/HSP induction has been mild, if even present (Pulliam et al., 2014; Seiferling et al., 2016), leaving the role of UPR<sup>mt</sup> in primary mitochondrial diseases open.

Mitochondrial myopathy (MM) manifests as progressive muscle weakness and respiratory chain (RC) dysfunction, typically caused by mtDNA deletions or mtDNA expression defects (mtDNA maintenance, translation; MM denoting diseases associated with these defects from now on). MM mice present a robust transcriptional stress response with induction of AARE-regulated genes and major remodeling of whole-cellular anabolic pathways, including serine, glutathione, and purine biosynthesis (Bao et al., 2016; Kuhl et al., 2017; Nikkanen et al., 2016). This mitochondrial integrated stress response (ISR<sup>mt</sup>), with metabolic and transcriptional components, is regulated by mTORC1 (mechanistic target of rapamycin C1), a major signal and growth integrator kinase (Khan et al., 2017; Zhang et al., 2013). In mammals, ISR<sup>mt</sup> is a robust response to mtDNA stress, integrating mitochondrial and cytoplasmic metabolism to pathways of growth and repair.

One of the most upregulated ISR<sup>mt</sup> components in humans and mice with MM is fibroblast growth factor 21 (FGF21). FGF21 has manifold roles in regulation of lipid and glucose metabolism, getting activated by low blood lipids and glucose, inducing ketone body synthesis, lipolysis and browning in white adipose tissue (WAT), improving glucose tolerance and regulating food intake (Badman et al., 2007; Inagaki et al., 2007; Kharitonov and Shanafelt, 2009; Kharitonov et al., 2005; Potthoff, 2017). So far, however, MM diseases are the only group of inherited diseases that consistently induces FGF21 in a genotype and phenotype specific manner. In mice and humans with MM, FGF21 is chronically secreted from the affected muscle fibers and/or heart independent of the feeding state (Agnew et al., 2018; Dogan et al., 2014; Keipert et al., 2014; Lehtonen et al., 2016, b; Ost et al., 2016; Pereira et al., 2017; Suomalainen et al., 2011; Tynysmaa et al., 2010). Interestingly, however, the response is specific to mtDNA expression diseases, whereas structural defects of the RC enzymes do not elicit a similar response (Lehtonen et al., 2016). These data indicate that different types of mitochondrial dysfunction induce different stress responses.

Here, we report that mammalian ISR<sup>mt</sup> progresses in temporal stages, and its components vary in different tissue types and disease stages, in post-mitotic or proliferating cell types. We show that FGF21 orchestrates progression of ISR<sup>mt</sup> and MM into systemic disease, with major effects for glucose and lipid metabolism, weight loss and brain manifestation of MM. Our data indicate that mitochondrial stress responses are 1) only partially conserved in species, 2) not generalizable between organisms or even mammalian cell types, and that 3) FGF21 is a key mediator of metabolic remodeling and progression of ISR<sup>mt</sup> locally and systemically.

## Results

### ISR<sup>mt</sup> progresses in temporal stages, involves different ATFs at different disease stages, and is partly distinct from UPR<sup>mt</sup> and UPR<sup>er</sup>

The various arms of ISR<sup>mt</sup> [UPR<sup>mt</sup>, activating transcription factor (ATF)/AARE, endocrine factors, metabolic remodeling] prompted us to ask, whether the different components are induced in all-or-none, parallel or sequential manner in MM mice, *in vivo*. We utilized the Deletor mice that show robust ISR<sup>mt</sup> (Khan et al., 2017; Nikkanen et al., 2016; Tynismaa et al., 2010) despite mild MM findings and normal life span (Tynismaa et al., 2005). The Deletors accumulate mtDNA deletions and show progressive RC deficiency in skeletal muscle and heart, as a consequence of ubiquitous expression of a dominant patient mutation in Twinkle, nuclear-encoded replicative helicase of mtDNA. These mice offer an excellent opportunity to dissect the long-term stress response dynamics upon MM progression. The first ISR<sup>mt</sup> signs in Deletor muscle coincide with the initial histological signs of RC deficiency [mosaic partial or complete cytochrome-c-oxidase (COX) deficiency] in <12-month-old mice and involve highly activated transcription of *Fgf21* and *Mthfd2* as well as *Gdf15* (Figure 1A, B). At 14-16 months, some COX-deficient fibers start to show increased mitochondrial mass and activity of succinate-dehydrogenase (SDH, RC complex II, exclusively nuclear-encoded and therefore not affected by mtDNA deletions) (Figure 1B). At this time, the second phase of ISR<sup>mt</sup> involves activation of *Atf5*, the regulatory enzymes of *de novo* serine biosynthesis [phosphoglycerate dehydrogenase (*Phgdh*), phosphoserine aminotransferase 1 (*Psat1*)] (Figure 1A) as well as mTORC1 (elevated phosphorylation of ribosomal S6 protein) (Figure 1B). These results indicate that ISR<sup>mt</sup> induction progresses in orderly temporal stages.

We then asked whether UPR<sup>mt</sup> is part of ISR<sup>mt</sup> in mammals. In Deletors of >21 months of age, mitochondrial HSP70 transcripts increase slightly, while mitochondrial HSP10 and HSP60 remain unchanged (Figure 1A). In >24-month-old Deletor muscle LonP1 (Lon peptidase 1), HSP60 and HSP10 protein amounts are increased in mitochondrial extracts, while HSP70 and ClpP (Caseinolytic mitochondrial matrix peptidase) are WT-like (Figure 1C, D). At this late stage, *Atf3* gets induced (Figure 1A). These data suggest that the affected skeletal muscle manifests a sequential ATF response, *Atf5* being progressively induced from the 1<sup>st</sup> to 2<sup>nd</sup> ISR<sup>mt</sup> stage and leading the response, and *Atf3* participating in terminal stage along with UPR<sup>mt</sup>. Despite non-significant marginal changes in *Atf4* transcripts, we cannot exclude its post-translational induction in the muscle. In conclusion, ISR<sup>mt</sup> in mammalian muscle proceeds in three stages that follow disease severity with initial activation of MTHFD2, FGF21 and GDF15, secondary induction of mTORC1, transsulfuration and serine biogenesis, followed by terminal-stage mild UPR<sup>mt</sup> (Figure 1E).

Optic atrophy 1 (OPA1) dependent membrane remodeling is commonly described as part of mitochondrial stress responses in cell culture and mitochondrial protein knockout mice (MacVicar and Langer, 2016; Olichon et al., 2003; Richter et al., 2013; Richter et al., 2015; Richter et al., 2019). In 24 month-old Deletors with advanced MM, the long isoform of OPA1 remains unprocessed (Figure 1C), showing that OPA1 processing is not an integral part of MM-related ISR<sup>mt</sup>. ISR<sup>mt</sup> is also distinct from endoplasmic reticulum (ER) stress (Gardner et al., 2013; Sidrauski and Walter, 1997; Taylor and Dillin, 2013), based on WT-like binding-immunoglobulin protein (BiP), X-box binding protein 1 (XBP1) splicing, and *Atf6* expression in the aged Deletor muscle (Figure S1A-C).

We then asked whether ISR<sup>mt</sup> induction in the muscle sends stress-signals to non-affected tissues, similar to UPR<sup>mt</sup> in *C. elegans* (Durioux et al., 2011), but found no evidence for such an event. The key ISR<sup>mt</sup> components are upregulated only in the primarily affected tissues with mtDNA deletions

(muscle, heart, BAT; Figure 1A, F), but not e.g. in the liver, a key metabolic organ. Therefore, unlike in the worm, ISR<sup>mt</sup> does not spread *per se* in mammalian tissues via a secreted factor.

### **ISR<sup>mt</sup> is conserved in human patients with mtDNA deletions**

FGF21 shows high concentrations in the serum and muscle of MM patients, compared to different other mitochondrial or non-mitochondrial muscle diseases (Lehtonen et al., 2016; Suomalainen et al., 2011). To examine, which parts of ISR<sup>mt</sup> are conserved from mice to men, we analyzed muscle biopsy samples of patients with MM, caused either by a single heteroplasmic mtDNA deletion or multiple mtDNA deletions, the latter caused by a dominant TWINKLE mutation, homologous to that in Deletor mice [Table 1, clinical details in (Spelbrink et al., 2001; Suomalainen et al., 1992; Suomalainen et al., 1997)]. The full ISR<sup>mt</sup> found in Deletor mice is also activated in human patients, including *MTHFD2*, *FGF21*, *GDF15*, *PHGDH*, *PSAT1*, and *SHMT2* (mitochondrial serine hydroxymethyl-transferase-2) as well as ATF-regulated *ASNS* (asparagine synthetase) and *TRIB3* (tribbles pseudokinase 3) (Figure 1G) and UPR<sup>mt</sup>-linked transcription factor *DDIT3* (DNA-damage-inducible transcript 3). However, all mitochondrial HSPs or proteases remain uninduced (Figure 1G). These results indicate robust conservation of the full ISR<sup>mt</sup> in mammals, from mice to human patients.

### **ISR<sup>mt</sup> progresses in temporal stages in proliferating cells after inhibition of mitochondrial translation**

To ask whether the temporal induction of ISR<sup>mt</sup> is induced in proliferating cells similar to muscle, we reversibly inhibited mitochondrial (mt) translation in mammalian diploid myoblasts. Actinonin is our inhibitor of choice, because 1) it leads to acute reversible down-regulation of mt-translation and ISR<sup>mt</sup>-like response (Richter et al., 2013; Richter et al., 2015)(Figure S1D) multiple mtDNA deletions do not accumulate in proliferating cells; C) both mtDNA deletion and mt-translation defects induce FGF21 as a sign of ISR<sup>mt</sup> (Lehtonen et al., 2016). We show that ISR<sup>mt</sup> components are robustly induced in mouse myoblasts, following the decrease of mt-translation from the early time points (1.5-2.5 h of actinonin exposure) (Figure 2A). This response is led by >40-fold induction of *Fgf21* transcription, accompanied by *Gdf15*, *Mthfd2* and cystathionine gamma lyase (CTH; both mRNA and protein), a regulatory enzyme of transsulfuration pathway (Figure 2B, C). Extended translational stress after time point 4.5 h, the 2<sup>nd</sup> ISR<sup>mt</sup> stage upregulates *de novo* serine biosynthesis enzymes, together with mitochondrial HSPs (Figure 2B, D). These data indicate that the acute response of mammalian diploid proliferating cells to mt-translation stress is orderly and sequential, similar to the post-mitotic tissues. However, different from MM-muscle, mt-translation defect ISR<sup>mt</sup> involves activation of OPA1 cleavage (Figure 2C) and induction of HSPs, indicating cell-type-specificity in responses to mtDNA expression defects.

### **ATF4 is required for ISR<sup>mt</sup> in dividing cells**

We then examined the contribution of the different ATFs in mouse myoblast ISR<sup>mt</sup>. Out of ATFs 1-7, the expression of *ATF3*, *ATF4* and *ATF5* get progressively induced in mouse myoblasts upon decreasing mt-translation, whereas other *ATFs* remain uninduced (Figure 2E). Mt-translation inhibition increases nuclear localization of ATF4 (Figure 2F), as a sign of its transcriptional activation. To clarify the hierarchy of the ATFs, we silenced ATF3, ATF4 and ATF5 one by one. We show that ATF4 is an upstream of regulator of *CTH*, *PHGDH*, *MTHFD2*, *GDF15* and *FGF21*, as well as *ATF3* (Figure 2G). However, *ATF5* activation is independent of ATF4, which is consistent considering the early and progressive induction of ATF5 also in the skeletal muscle, without considerable activation of ATF4 at the time (Figure 1A). Also, mitochondrial fragmentation is not affected by ATF4 silencing (Figure 2F), suggesting its regulation by an ISR<sup>mt</sup>-independent mechanism. ATF3 or ATF5 silencing indicates that

they are not essential for ISR<sup>mt</sup> induction in proliferating cells. The combined evidence from muscle and proliferating cells suggest that ATF5, ATF4 and ATF3 all take part in ISR<sup>mt</sup>, with cell-type and stress specificity, finetuning metabolism in acute and chronic mtDNA expression stress.

### **FGF21 modulates local and systemic metabolism in MM**

Our findings above show that FGF21 is an early responder to mtDNA replication and translation stress both in post-mitotic tissues and proliferating cells. To assess FGF21 contributions to disease progression and ISR<sup>mt</sup>, we generated Deletor mice lacking FGF21. In short, Deletors with Floxed *Fgf21* (FGF21<sup>LoxP/LoxP</sup>) (Potthoff et al., 2009) were crossed with mice constitutively expressing *cre*-transgene (PGK-promoter) (Lallemand et al., 1998) to generate full-body FGF21 knockout Deletors (DEL-FKO) (Figure S3A). DEL-FKO mice are unable to induce *Fgf21* expression in the affected muscle or heart, or from the liver during fasting (Figure 3A-C; S3B) but their viability is WT-like in 24 months of follow-up.

Body weight of Deletors declines progressively from 16-18 months of age (Figure 3D), coinciding with the time when FGF21 becomes detectable in the blood (Figure S3C). However, *Fgf21* transcript level is high in Deletor muscle already at 12 months of age, progressively increasing from the early manifestation of RC deficiency to ~25-50 fold compared to WT (Figure S3C). FGF21 knockout prevents weight loss, normalizes subcutaneous adipocyte size and prevents white adipose tissue (WAT) browning of Deletor mice (Figure 3D, E). Deletor liver fat is low, the small fat droplets typically residing intracellularly, whereas in WT and FKO mice liver fat is mostly extracellular. DEL-FKO liver shows WT-like extracellular fat content, whereas FKO in healthy background leads to excessive fat accumulation (Figure 3F). These data suggest that systemic FGF21 has manifold consequences to liver and adipose tissue fat content in MM and explains the low body weight – a symptom typical for mitochondrial disease patients.

To obtain insight into FGF21 roles in systemic metabolism, we performed *in vivo* uptake assays of glucose and fatty acid analogues (<sup>18</sup>F-fluorodeoxyglucose, <sup>18</sup>F-FDG; <sup>18</sup>F-Fluoro-6-thia-heptadecanoic acid, <sup>18</sup>F-FTHA). The affected muscle, heart and beige/brown subcutaneous WAT of Deletors show significantly increased <sup>18</sup>F-FDG uptake (Figure 3G), normalized in DEL-FKO, indicating that FGF21 drives glucose uptake in the affected tissues in MM (Figure 3G). Fatty acid uptake (palmitic acid; saturated 16-carbon fatty acid) is not changed in Deletor or DEL-FKO mice (Figure 3H). Metabolic cage examination of systemic metabolism in Deletors (ambient temperature and thermoneutral +30 °C) shows high oxygen consumption, CO<sub>2</sub> production and slightly increased respiratory exchange ratio compared to WT mice, consistent with glucose usage as main fuel (Figure 3I, J; S3D). In DEL-FKO mice, however, metabolism was WT-like. These results indicate that glucose is the major metabolic fuel in MM and the disease-driven high glucose uptake into the affected tissues is FGF21-dependent.

### **FGF21 has no effect on MM disease signs**

The tight connection between FGF21 and advancing disease made us to ask whether loss of FGF21 affected the pathologic hallmarks of MM: the RC deficient muscle fibers, mtDNA deletions, mitochondrial mass and RC protein amounts (Figure 3K-N, S3E, F). None of these disease signs were affected by FGF21 inactivation, demonstrating that FGF21 does not contribute to development of mtDNA deletions, mitochondrial biogenesis or RC deficiency in skeletal muscle.

### **FGF21 regulates serine biosynthesis and transsulfuration in MM**

*Fgf21* is a major component in the first stage ISR<sup>mt</sup> induction in the skeletal muscle of Deletor mice, and also one of the most highly increased transcripts in the human patient muscle. Therefore, we investigated the role of FGF21 in the progression of local muscle ISR<sup>mt</sup> in MM. Intriguingly, induction of the 2<sup>nd</sup> ISR<sup>mt</sup> stage is completely dependent on the presence of FGF21: *Atf3* and *Atf4*, serine *de novo* biosynthesis (*Phgdh* and *Psat1*) and transsulfuration (CTH) all are WT-like in DEL-FKO mice (Figure 4A, B). mTORC1 activation, however, is FGF21-independent, remaining active in RC-deficient fibers (Figure S4A). The components of the 1<sup>st</sup> ISR<sup>mt</sup> stage, activated at the same time as FGF21, are unaffected by FGF21KO (*Mthfd2*, *Atf5*; ATF-regulated genes *Trib3* and *Asns*; *GDF15*) (Figure 4A, B). The data imply differential roles for different ATFs: *Atf3* and *Atf4* upregulation in the 2<sup>nd</sup> ISR<sup>mt</sup> stage is dependent on FGF21, whereas progressive induction of *Atf5*, starting at the 1<sup>st</sup> ISR<sup>mt</sup> stage is independently regulated in MM muscle. We also report uncoupling of regulation of mitochondrial folate cycle: MTHFD1L induction in Deletors is FGF21-dependent, whereas MTHFD2 is not (Figure 4B).

In cultured cells with mt-translation inhibition (Figure 2B) FGF21 was an early 1<sup>st</sup> ISR<sup>mt</sup> responder, raising the question whether it regulates downstream ISR<sup>mt</sup> also in these cells. However, FGF21KO fibroblasts respond to actinonin similar to WT-cells: both transsulfuration and *de novo* serine biosynthesis remain induced (Figure 4SB). This suggests that the role of FGF21 as a regulator of ISR<sup>mt</sup> progression is either stress-signal or cell-type specific. Altogether, these results show that auto/paracrine FGF21 is essential for ISR<sup>mt</sup> progression and metabolic remodeling from 1<sup>st</sup> to 2<sup>nd</sup> stage, from early to chronic, in postmitotic skeletal muscle in MM.

As the metabolic effects of FGF21 involved biosynthetic pathways via folate-driven one-carbon cycle, we analyzed folate forms and targeted metabolome of ~100 metabolites representing the pathways of interest (Figure S4C). 5-methyl-tetrahydrofolate (THF) is rate-limiting for methyl cycle and provides one-carbon units and precursors for transsulfuration and methylation reactions. In Deletor muscle, 5-methyl-THF, betaine, choline, and glutamate required for methyl cycle and transsulfuration show high concentrations, but in DEL-FKO their levels are WT-like (Figure 4C; S4C). Figure 4D summarizes the key changes in transsulfuration between Deletors and DEL-FKO. These data suggest that 1) FGF21 drives cell-autonomous metabolic remodeling in MM, involving major biosynthetic pathways; 2) FGF21-independent MTHFD2 (mitochondrial one-carbon cycle) does not contribute to cytoplasmic one-carbon cycle changes in MM muscle.

Amino acids show a general increase in Deletor muscle, and these levels shift to WT-direction in DEL-FKO (Figure 4E). Asparagine is an exception, remaining high in DEL-FKO, consistent with increased expression of its synthesizing enzyme *Asns* in both Deletors and DEL-FKO, and suggesting glutamine usage of Deletor muscle (Zhang et al., 2014). In DEL-FKO, long-chain acylcarnitines accumulated (Figure 4F, S4C) despite the fact that their uptake was not increased (Figure 3H) suggesting inability to oxidize fatty acids in MM.

Imbalance of nucleotide metabolism intermediates and dNTPs is a metabolic hallmark of MM (Nikkanen et al., 2016), purine biosynthesis being one of the key pathways dependent on mitochondrial folate cycle (French et al., 2016). However, dNTP pools in Deletors are not improved by absence of FGF21 (Figure 4G); actually, we show trend toward elevated dNTPs and their precursors also in FKO, including deoxyuridine (Figure S4C). The data suggest that FGF21 has an overall inhibitory effect on dNTP homeostasis, even in normal muscle.

Altogether, our evidence indicates that auto/paracrine FGF21 remodels the major anabolic biosynthesis pathways, the cytoplasmic one-carbon cycle, in MM muscle (summarized in Figure 4H).

## FGF21 drives glucose uptake and mitochondrial proliferation in dorsal hippocampus in MM

FGF21 mediates signals of nutrient metabolism from peripheral organs to the brain, via binding to  $\beta$ -Klotho receptors in the choroid plexus and hypothalamus in WT-mice (Kurosu et al., 2007; Ogawa et al., 2007; Potthoff, 2017). However, little information exists of the effects of periphery-derived FGF21 on CNS metabolism in disease. Remarkably, Deletor brain shows increased *in vivo* uptake of  $^{18}\text{F}$ -FDG specifically in dorsal hippocampus (DHC; CA2 region). This glucose uptake is fully FGF21-dependent (Figure 5A, B; S5A). *Fgf21* transcription is not locally elevated in DHC or any region of Deletor brain (Figure 5C) suggesting that periphery-derived endocrine FGF21 mediates glucose-uptake in DHC in MM. To clarify whether DHC glucose uptake reacts to FGF21 also in WT mice, we induced FGF21 expression by fasting (24 h) or by intravenous injection of recombinant FGF21, raising FGF21 serum levels 4-16 -fold higher than in WT, respectively (Figure S5B). However, these interventions did not affect WT DHC glucose uptake (Figure S5B). These data indicate that high glucose uptake in DHC is a manifestation of MM.

Despite the lack of apparent nervous system symptoms, MM patients and Deletor mice both show mtDNA deletions in their brain (Suomalainen et al., 1992; Tynismaa et al., 2005). DEL-FKO mice harbor similar amounts of mutant mtDNA in their DHC as the Deletors. Unlike in the muscle, however, mTORC1 activity (S6-phosphorylation) is not increased in the hippocampus of aged Deletors or DEL-FKO (Figure 5E). This conclusion is further supported by the fact that inhibition of mTORC1 activity by rapamycin does not affect *in vivo* glucose uptake in DHC (Figure S5A), indicating cell-specific roles of mTORC1 in MM.

Unlike in the Deletor muscle, mitochondrial RC activities and mitochondrial proliferation in DHC remarkably depend on presence of FGF21. SDH activity is increased in Deletor CA2 of DHC, especially in the neuronal projections spanning all the layers of CA2, while the neighboring CA1, CA3 and dentate gyrus are WT-like (Figure 5F, G). CA2 also shows low COX-amount (Figure 5H), consistent with typical RC deficiency. Surprisingly, however, both the upregulated SDH activity and COX-protein were WT-like in DEL-FKO mice, suggesting that and FGF21-dependent mechanism promotes RC deficiency in CA2 of DHC (Figure 5F-H). Immunohistological analysis of SDHA and mitochondrial outer membrane protein TOM20 confirm the increase of mitochondrial mass in CA2 neurons of Deletors, and WT-like mitochondrial mass in DEL-FKO (Figure 5I, J). Similar dendritic and soma appearance of pyramidal CA2 neurons and lack of astrogliosis or inflammation suggest no major neuronal damage or cell loss in Deletors or DEL-FKO (Figure 5K; S5D, E). These data suggest an intriguing possibility that muscle-derived FGF21 affects RC function and mitochondrial biogenesis in the brain in MM.



## Discussion

Knowledge of the dynamics, regulation and tissue-specific characteristics of mammalian stress responses to mitochondrial disease has been insufficient. Here, we report that mitochondrial integrated stress response (ISR<sup>mt</sup>) is temporal and stage-wise orchestrated in mice and humans with mtDNA stress in skeletal muscle. The early ISR<sup>mt</sup> is cell-autonomous with activated expression of *FGF21*, *GDF15*, mitochondrial one-carbon cycle and increasing expression of *ATF5*. The 2<sup>nd</sup> disease stage activates serine and glutathione synthesis, glucose uptake into affected tissues and systemic metabolic changes, and the 3<sup>rd</sup> terminal stage shows slight activation of *ATF3* and mitochondrial HSPs. Intriguingly, we show that progression of ISR<sup>mt</sup> to 2<sup>nd</sup> stage is dependent on metabolic hormone FGF21, which has major local and systemic consequences on metabolism, including in the brain. These results emphasize cross-talk of the affected and distant organ systems in mitochondrial disease and indicate that a primary mitochondrial muscle disease has major systemic consequences.

Mitochondrial stress responses have been predominantly characterized in the worm *C. elegans*, in which RNAi ablation of RC subunits induced an UPR<sup>mt</sup> pathway as a consequence of nuclear relocation of ATFS-1, activating expression of mitochondrial HSPs and proteases (Durieux et al., 2011; Haynes et al., 2007; Nargund et al., 2012). In mammalian cancer cells, mitochondrial expression of an aggregation-prone protein elicits a UPR<sup>mt</sup>-like response (Zhao et al., 2002), as also occurs in knockout-mice for essential mitochondrial proteins (Bao et al., 2016; Ost et al., 2015; Pulliam et al., 2014; Seiferling et al., 2016). We previously described ISR<sup>mt</sup> as the major stress response in mammalian MM. The response involved a wide transcriptional and metabolic remodeling beyond UPR<sup>mt</sup> (Khan et al., 2017; Nikkanen et al., 2016), also found in other models with mtDNA maintenance and expression defects (*Twnk*, *Tfam*, *Polrmt*, *Lrpprc*, *Mterf4*) (Kuhl et al., 2017). Here, we show that already the first subclinical disease signs – single COX-negative muscle fibers in 10-12-month-old Deletor mice – activate local expression of endocrine factors FGF21 and GDF15, as well as mitochondrial one-carbon cycle. *Fgf21* transcription is first highly upregulated in the muscle, but the protein is increased in the blood only after 4-6 months, at the same time when ragged-red fibers – a hallmark of MM – emerge in the muscle. FGF21 presence in the blood induces weight loss and WAT browning, known endocrine consequences of liver-derived FGF21 in fasting rodents (Badman et al., 2007; Xu et al., 2009). In MM muscle, the 2<sup>nd</sup> ISR<sup>mt</sup> stage activates only if FGF21 is present, with upregulated glucose uptake, *de novo* serine biogenesis, and transsulfuration. Therefore, the local and systemic 2<sup>nd</sup> ISR<sup>mt</sup> stage is crucially dependent on auto/para- and endocrine effects of FGF21, which together mark the progression of the muscle disease from local to systemic. Which of the FGF21-dependent metabolic events are direct consequences of the hormone, remains to be studied.

FGF21-independent components of the second ISR<sup>mt</sup> stage in the muscle include mTORC1 activation, nucleotide pool imbalance and mitochondrial mass increase, agreeing with the previous data of mTORC1 contribution in RRF formation (Khan et al., 2017), and the regulatory role of mTORC1 for purine synthesis in cancer cells (French et al., 2016). In the hippocampus of MM mice, mitochondrial mass increase or RC deficiency are dependent of periphery-derived FGF21, but mTORC1 is not active, emphasizing tissue-specificity of the responses. Overall, these data indicate that mtDNA expression defects induce an early, local subclinical response, while a second systemic, chronic stage modifies metabolism in the whole organism. These data underline the complexity of ISR<sup>mt</sup> and the essential roles of FGF21 both in the dynamics of ISR<sup>mt</sup> and progression of MM to a systemic condition.

Subtle induction of UPR<sup>mt</sup>-related mitochondrial HSPs occurs in two-year-old Deletor mice, forming the third ISR<sup>mt</sup> stage. In mammals, this HSP response does not spread beyond the affected tissue, unlike in *C. elegans* (Durieux et al., 2011; Zhang et al., 2018). Still the endocrine effects of muscle-derived FGF21 on hepatic lipolysis, WAT browning, brain glucose uptake and mitochondrial mass and

function mimic the “mitokine” concept of the worm: FGF21 spreads a signal of mitochondrial dysfunction from the affected organ to distant ones. The exact mechanism of how the FGF21-related muscle-to-brain signaling occurs in our mice, specifically affecting the dorsal hippocampus, requires future attention, as this region has not previously been associated with metabolic regulation or mitochondrial disease. We conclude that endocrine signaling induced by mtDNA stress, from the affected to distant tissues, is conserved from worms to mammals but in mammals is importantly contributed by FGF21, signaling from the periphery to the brain.

FGF21 had differential effects of RC deficiency and mitochondrial mass in the muscle and DHC. The dorsal hippocampus of Deletor mice showed mitochondrial mass increase and RC deficiency only in the presence of FGF21, but no changes in mtDNA deletion load. In the muscle, however, FGF21 did not affect RC activities or mitochondrial mass. mTORC1 is induced in muscle fibers with mitochondrial proliferation, but in the brain of the same animals, mTORC1 is not activated. These data underscore the remarkable tissue specificity in the upstream regulation of the mitochondrial stress responses. Importantly, our evidence indicates that a “general mitochondrial stress response” does not exist, but the specific components of ISR<sup>mt</sup> need to be characterized separately for different organisms, tissues and insults.

ATF transcription factor family regulates the AARE-response, but which homologues of the worm ATFS-1 are important in mammals, has been controversial. Previously, toxin-mediated oxidative stress (paraquat) in human embryonic kidney cells was reported to induce mitochondrial HSPs in an ATF5-dependent manner (Fiorese et al., 2016). Thereafter, a multi-omics approach identified ATF4 to be the main driver of ISR<sup>mt</sup> in cervical cancer cells treated with variable inhibitors of mtDNA expression, mitochondrial protein import or membrane potential (Quiros et al., 2017). Our data from MM mice show involvement of ATF5 and ATF3 in post-mitotic muscle, with different temporal dynamics. ATF5 expression is a robust early responder to mtDNA replication stress in both mice and human patients. In mice, ATF3 (and 4) get induced in the later stages. Interestingly, upregulation of ATF3 and ATF4 depend completely on the presence of FGF21, whereas ATF5 is FGF21-independent, further emphasizing the chronology of induction. Their roles are, however, different in proliferating cells where ATF4 gets activated early, is FGF21-independent and required for the activation of other ISR<sup>mt</sup> components. These data indicate that the ATF-family is well conserved as a mitochondrial stress sensor from worms to mammals, but specific ATFs have different roles in different cell types and in acute or chronic stress.

In Deletors the overall health, muscle morphology or lifespan are not significantly affected by the presence or absence of FGF21, and they show no signs of inflammation; the major effects of FGF21 are metabolic. These findings differ from those in muscle-specific OPA1-KO mice, in which FGF21 induction was deleterious, promoting inflammation (Tezze et al., 2017), which may be explained by the difference in severity of the mitochondrial insult in OPA1-KO versus Deletor mice. Embryonic OPA1-KO in muscle is lethal at early postnatal life and even conditional OPA1-KO in muscle results in severe muscle atrophy, rapid body weight loss and death of the mice <100 days after induction, reflecting the vital nature of the protein (Tezze et al., 2017). Remarkably, FGF21KO in OPA1-KO animals reverted the systemic inflammation and prolonged the lifespan for >100 additional days. The results from OPA1-KO and Deletors together suggest concentration- and/or context-dependence of FGF21 effects, which may have clinical relevance: children with severe, often early lethal disorders of mt-translation or mtDNA maintenance have very high FGF21 concentration (up to 200-fold), whereas adults with MM show more modest induction (2 to 10-fold) (Lehtonen et al., 2016) – the former mimic FGF21 in OPA1-KO, and the latter FGF21 in Deletors. These data suggest that upon severe mitochondrial dysfunction,

inhibition of FGF21 might be beneficial, whereas in late-onset disorders, the mildly elevated FGF21 driving e.g. glutathione synthesis might be protective.

Importantly, our evidence indicates that mammalian ISR<sup>mt</sup> is a complex response, with coordinated dynamics, auto/paracrine feedback loops and endocrine signaling, affecting the whole organism, with FGF21 as its circulating messenger. The initial signal(s) that trigger the early ISR<sup>mt</sup> remain to be studied. Previous studies in cultured cells suggested the signal to involve accumulation of aberrant mt-translation products in the inner mitochondrial membrane (Richter et al., 2015). Our results highlight the systemic metabolic consequences of mitochondrial dysfunction that extend far beyond the primary RC deficiency or the affected organ. The usefulness of FGF21 as a therapy target remains to be studied: despite the robust morphologic and metabolic changes, the functional defects of the Deletor mice with and without FGF21 are subtle and their lifespan normal. However, the FGF21 contribution to weight-loss might be relevant also for patients, as this is one of their most common and socially challenging symptoms. Furthermore, our results indicate that a muscle disease can affect brain metabolism, highlighting the importance of zooming out from specific tissues when considering disease pathogenesis and considering the whole organism when assessing treatment effects.

### **Limitations of Study**

Twinkle dysfunction in the Deletor-mouse and patients with mitochondrial myopathy causes progressive accumulation of mtDNA deletions in post-mitotic tissues. MtDNA deletions are typically not produced/maintained in replicating cell types, and the mechanistic overlap of the mitochondrial stress response in postmitotic tissues and our cultured cell model for mt-translation stress may only be partial.

The Deletor and Deletor-FGF21KO mice have a normal lifespan and show no motor deficiency. Therefore, the effects of FGF21 or absence for the performance of the mitochondrial myopathy mice could not be tested. 3) The evidence provided by the full-body Deletor-FGF21KO mice undisputedly shows that FGF21 is a required for the metabolic phenotypes in the Deletors, especially in the muscle and brain. However, whether these phenotypes are directly FGF21-dependent, or mediated by a signaling cascade, remains to be studied.

## Acknowledgments

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## Author Contributions

S.F. designed and conducted *in vivo* study, analyzed and interpreted data; C.J. designed and conducted *in vitro* study, analyzed and interpreted data. C.J.C., M.K., J.N and L.E. participated in study design and supervision; I-M.K., S.P., A.M., N.K. P.M., H.L. conducted experiments, analyzed and interpreted data. L.W.: expertise in nucleotide analysis and interpretation. E.P. and M.A. collected and analyzed patient data. U.R. and B.J.B. participated in study design and interpretation. A.R. supervised PET analyses, and V.V. supervised metabolomics analysis and interpretation. A.S. conceived, designed and supervised the study. S.F., C.J. and A.S. wrote the manuscript, which was edited and approved by all co-authors.

## Declaration of Interests

The authors declare no competing interests.

## Main Figure Titles and Legends

### Figure 1. ISR<sup>mt</sup> progresses in defined temporal stages in muscle and is conserved in mouse and man.

**(A)** Chronological progression of ISR<sup>mt</sup> in MM muscle. 1<sup>st</sup> stage: metabolic hormones (*Fgf21*, *Gdf15*) and mitochondrial folate cycle (*Mthfd2*). 2<sup>nd</sup> stage: *de novo* serine biosynthesis (*Phgdh*, *Psat1*). 3<sup>rd</sup> stage: mitochondrial HSPs. ATFs 3-5 activate at different time-points. FC: mRNA against beta-actin mRNA. Details and statistical analysis see Table S1.

**(B)** Progression of RC enzyme deficiency and mTORC1 activation in MM muscle. Top: histochemical *in situ* enzyme activity of COX (brown) and SDH (blue). White fibers: COX-negative. Blue fibers: COX-negative, SDH positive. Bottom: phosphorylated ribosomal S6 protein (pS6 immunohistochemistry). Subsequent frozen sections; lines and arrows indicate same fibers. Scale bar 50  $\mu$ m.

**(C-D)** UPR<sup>mt</sup> associated HSPs and proteases in skeletal muscle mitochondrial extracts; western blot. Porin and TOM20: loading controls, quantification of signals against porin in (D), n=5 per group.

**(E)** Schematic representation of the temporal ISR<sup>mt</sup> stages of MM.

**(F)** ISR<sup>mt</sup> or UPR<sup>mt</sup> transcript induction in different tissues compared to beta-actin mRNA, n=6 per group.

**(G)** ISR<sup>mt</sup> induction in human patients with MM. Table 1 and Table S2 summarize subjects and details of transcript levels results, respectively.

Animal ages: 22-24 months. Bars: average and standard deviation. Box: 25<sup>th</sup> and 75<sup>th</sup> percentiles with median and range. Dots: individual subjects. Pairwise two-tailed t-test, statistical significance: \*p $\leq$ 0.05, \*\*p $\leq$ 0.01, \*\*\*p $\leq$ 0.001, \*\*\*\*p $\leq$ 0.0001. Abbreviations: ASNS=asparagine synthetase, ATF3-5=activating transcription factors, BAT=brown adipose tissue, CLPP=caseinolytic mitochondrial matrix peptidase, COX=cytochrome *c* oxidase, DDIT3=DNA-damage inducible transcript-3, FC=fold change, FGF21=fibroblast growth factor 21, GDF15=growth and differentiation factor 15, HSP10/60/70=mitochondrial heat shock proteins 10/60/70, LONP1=lon peptidase 1, MTHFD2=methylene-tetrahydrofolate-dehydrogenase-2, mTORC1=mechanistic target of rapamycin C1, OPA1=optic atrophy 1, PHGDH=phosphoglycerate dehydrogenase, PSAT1=phosphoserine aminotransferase-1, SHMT1(2)=serine hydroxymethyltransferase 1 (2, mitochondrial), SDH=succinate dehydrogenase, TOM20=translocase of outer mitochondrial membrane 20, TRIB3=tribbles pseudokinase 3, WATip/sc=white adipose tissue intraperitoneal/subcutaneous.

**Figure 2. Temporal progression of ISR<sup>mt</sup> in proliferating cells after acutely inhibited mitochondrial translation**

**(A)** Mt-translation inhibition upon actinonin exposure; <sup>35</sup>S-Methionine/Cysteine incorporation assay and quantification of total signal versus total protein. Abbreviations: ND1-6=NADH dehydrogenase subunits 1-6 (Complex I), CyB=Cytochrome b (Complex III), Co1-3=Cytochrome-c-oxidase subunits 1-3 (Complex IV), ATP6, ATP8=ATP synthase subunits 6 and 8 (Complex V).

**(B)** Temporal transcriptional activation of ISR<sup>mt</sup> components upon mt-translation inhibition. Actinonin exposure in mouse myoblasts, mRNA quantified against beta-actin mRNA.

**(C)** OPA1 cleavage and progressive CTH induction upon actinonin exposure. Western blot analysis of protein extracts from mouse myoblasts, mitochondrial porin as a loading control.

**(D)** Temporal activation of mitochondrial HSP expression upon mt-translation inhibition; actinonin exposure, mouse myoblasts.

**(E)** Temporal activation of *ATF3*, *4* and *5* expression upon mt-translation inhibition; actinonin exposure, mouse myoblasts.

**(F)** Nuclear localization of ATF4 upon mt-translation inhibition. Immunofluorescence analysis of untreated (top), actinonin treated (middle) and actinonin+*ATF4* siRNA treated (bottom) human fibroblasts, scale bar 5  $\mu$ m.

**(G)** ISR<sup>mt</sup> induction upon mt-translation inhibition; actinonin exposure after siRNA-mediated silencing of *ATF3*, *4* or *5* in human fibroblasts.

**(H)** Schematic representation of ISR<sup>mt</sup> progression in growing cells, upon mt-translation inhibition.

Gene expression analyses in B, C, E and G represent consensus of minimum of three separate actinonin treatment experiments. Transcripts presented in B, C and E are measured from the same cells. Abbreviations (also see legend of Figure 1): Act=actinonin, CTH=cystathionine gamma-lyase, DAPI=4',6-diamidino-2-phenylindole (DNA dye), EtOH=ethanol.

**Figure 3. Muscle-derived FGF21 inhibits fat storage and induces glucose uptake without affecting pathological hallmarks**

**(A-C)** FGF21 expression in Deletor and FGF21 knockout lines; mRNA FC against beta-actin. (A) *Fgf21* in skeletal muscle, n=5 per group. (B) Serum FGF21; WT n=7, Deletor n=6; FKO and DEL-FKO n=3. (C) *Fgf21* expression in different tissues, compared to Deletor muscle, n=3 per group.

**(D)** Body weight of mice at the age of Deletor phenotype manifestation (11-24 months). WT, FKO n=8; Deletor and DEL-FKO n=7. Significance calculated against WT mice.

**(E)** Subcutaneous adipocyte size and browning. UCP1 immunohistochemistry (scale bar 25  $\mu$ m) and hematoxylin-eosin staining (scale bar 100  $\mu$ m).

**(F)** Lipid droplets in liver, Oil-Red-O staining for lipids and triglycerides, scale bar 40  $\mu$ m.

**(G-H)** *In vivo* uptake of glucose ( $^{18}$ F-FDG) and fatty acid ( $^{18}$ F-FTHA, C16:0) analogs in tissues. Statistical analysis and details in Table S3.

**(I-J)** Respiratory exchange ratio (RER,  $v\text{CO}_2/v\text{O}_2$ ) in thermoneutral condition (+30  $^{\circ}$ C). RER  $\sim$ 1: preferred fuel carbohydrates; RER  $\sim$ 0.7 lipids as fuel. Light = sleep; dark = active. (J) Average of RER when mice are active. WT, Deletor and DEL-FKO n=3 per group; FKO n=6.

**(K-N)** Hallmarks of MM in skeletal muscle. (K) Histochemical *in situ* RC enzyme activities (brown precipitate = COX-positive; white = COX-negative; blue = SDH-positive, COX-negative). Scale bar 100  $\mu$ m. (L) Quantification of (K). WT, FKO n=6, Deletor n=5, DEL-FKO n=8. (M) Long-range PCR of mtDNA. Smear: multiple deletions ( $\Delta$ mtDNA); arrow: full-length 16.6 kb mtDNA. Short range PCR of mtDNA: non-deleted D-loop region (573 bp). WT and FKO n=3; Deletor and DEL-FKO n=5. (N) Quantitative qPCR analysis of full-length mtDNA (ratio of ND4 gene region and non-deleted D-loop; n=5/group).

Animals: 22-24 months old. Bars: average and standard deviation. Box: 25<sup>th</sup> and 75<sup>th</sup> percentiles with median and range. Dots: individual subjects. Pairwise two-tailed t-test, statistical significance: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ . Abbreviations (also see legend of Figure 1): UCP1=mitochondrial uncoupling protein 1,  $^{18}$ F-FDG= $^{18}$ F-fluorodeoxyglucose,  $^{18}$ F-FTHA= $^{18}$ F-Fluoro-6-thia-heptadecanoic acid.

**Figure 4. FGF21 regulates induction of *de novo* serine biosynthesis and transsulfuration**

**(A)** Differential FGF21-dependence of ATFs and ISR<sup>mt</sup> expression in mouse muscle. Transcripts vs beta-actin mRNA (fold-change).

**(B)** Differential FGF21-dependence of mitochondrial folate cycle and transsulfuration. Representative western blot analysis of protein extracts from skeletal muscle, total protein signal as loading and quantification control. N=3/group.

**(C)** Dependence of methyl cycle linked metabolites on FGF21. Targeted metabolomics, significance calculated against WT. WT, FKO n=8, Deletor, DEL-FKO n=6.

**(D)** Schematic representation of transsulfuration pathway and linked metabolites, highlighting the FGF21-dependent changes in Deletor vs WT; DEL-FKO vs FKO. Red, increased; black, no change; grey, NA.

**(E-F)** Muscle amino acids and acylcarnitines. Targeted metabolomics, FC normalized to WT. Acylcarnitines shown in the order of increasing chain length [C, carnitine]. Significance calculated against WT. Statistical analysis and details in Tables S4 and S5.

**(G)** Tissue dNTP pools, FC normalized to WT, n=3/group.

**(H)** Schematic representation of folate-driven one-carbon cycle and associated biosynthetic pathways (nucleotide metabolism, methyl cycle, transsulfuration and *de novo* serine biosynthesis) altered in Deletor. Arrows point to FGF21 effects.

Animals: 22-24 months old. Bars: average and standard deviation. Box: 25<sup>th</sup> and 75<sup>th</sup> percentiles with median and range. Dots: individual subjects. Pairwise two-tailed t-test, statistical significance: \*p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001, \*\*\*\*p≤0.0001. Abbreviations (see also Figure 1): dNTP=deoxynucleotide, dTMP=deoxythymidine monophosphate, GCL=Glutamate-cysteine ligase, Hcy=homocysteine, GS=Glutathione synthetase, THF=tetrahydrofolate, SAH=S-adenosylhomocysteine, SAM=S-adenosylmethionine.



**Figure 5. FGF21 drives glucose uptake, increased mitochondrial mass and respiratory activity in CA2 of hippocampus**

**(A-B)** *In vivo* glucose ( $^{18}\text{F}$ -FDG) uptake in coronal brain sections; *ex vivo* digital autoradiography (ARG); photostimulated luminescence per unit area, PSL/mm<sup>2</sup>. (B) Quantification of (A), normalized to cerebellar cortex (similar cerebellar  $^{18}\text{F}$ -FDG uptake between the groups confirmed by manual gamma ray emission measurement; data not shown). WT, FKO, DEL-FKO n=6, Deletor n=5.

**(C)** *Fgf21* expression in different brain areas of our mouse strains. mRNA vs beta-actin mRNA. Positive controls: Deletor muscle and WT-mouse liver.

**(D)** Schematic illustration of hippocampus and CA2 in sagittal view of mouse brain. Hippocampal layers: so=stratum oriens, sp=stratum pyramidale, sr=stratum radiatum, slm=stratum lacunosum-moleculare.

**(E)** Phosphorylated S6, a downstream target of mTORC1. Immunohistochemical detection at CA1/CA2 border (dotted line); scale bar 100  $\mu\text{m}$ .

**(F-G)** Histochemical *in situ* enzyme activity of COX (brown = active) and SDH (blue = SDH positive/COX-negative). CA2 in Deletor: increased SDH activity, scale bar 200  $\mu\text{m}$ . (G) Enlarged image of CA2 (box) of (F), arrows point to increased SDH-activity in long projections (sr), scale bar 30  $\mu\text{m}$ .

**(H)** COX protein amount in hippocampus. Immunohistochemical detection of COX (MTCO1 antibody) in CA2 and CA1 border (dotted line). Encircled: pyramidal cell layer; Deletor, low COX-protein intensity in CA2, when compared to DEL-FKO and WT. Scale bar 100  $\mu\text{m}$ .

**(I-J)** Mitochondrial mass in CA2 and CA1 (border: dotted line). (H) SDH amount (SDHA antibody; increased staining in sr with dendritic extensions of pyramidal neurons). Scale bar 100  $\mu\text{m}$ . (I) Mitochondrial amount (TOM20 antibody) at CA2/CA1 border increased in extensions of pyramidal neurons (arrows, sr). Scale bar 50  $\mu\text{m}$ .

**(K)** Dendritic network in hippocampal CA2/CA1; MAP2 immunofluorescence. Scale bar 50  $\mu\text{m}$ .

Animals: 22-24 months old. Bars: average and standard deviation. Dots: individual subjects. Pairwise two-tailed t-test, statistical significance: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ . In histology, n=3 per group. Abbreviations (see also Figure 1 legend): cpu=caudoputamen/striatum, CTX=cortex, DG=dentate gyrus, DHC=dorsal hippocampus, HT=hypothalamus, MAP2=microtubule-associated protein 2, VHC=ventral hippocampus,  $^{18}\text{F}$ -FDG= $^{18}\text{F}$ -Fluorodeoxyglucose.

## Main Tables and Legends

**Table 1. Clinical characteristics of MM patients.**

	<b>Sex</b>	<b>Age at sampling</b>	<b>Age of disease onset</b>	<b>Disease cause</b>
Patient 1	F	57	33	Single mtDNA deletion
Patient 2	M	55	21	Multiple mtDNA deletions *
Patient 3	F	54	30	Multiple mtDNA deletions *
Patient 4	F	70	31	Single small mtDNA deletion
Controls n=8	6 F / 2 M	48-64		-

\*TWINKLE mutation leading to 13 amino acid insertion in linker domain = Homologous mutation to that in Deletor mouse.

## STAR METHODS

### LEAD CONTACT FOR REAGENTS AND RESOURCE SHARING

Requests and detailed information of reagents should be directed and will be made available by Anu Suomalainen (anu.wartiovaara@helsinki.fi).

### KEY RESOURCE TABLE

Resources and reagents are listed in the key resource table.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

#### Ethical approval

The National Animal Experiment Review Board and Regional State Administrative Agency for Southern Finland approved the animal experimentation, following the European Union Directive. The human materials were collected and used with informed consent according to the Helsinki Declaration and approved by the Ethical Review Board of Helsinki University Central Hospital.

#### Animal Models

The Deletor mice expressed a dominant in-frame duplication, homologous to a human mitochondrial myopathy mutation. Two previously reported transgene lines were used in this study. We generated the Deletor mouse with *Fgf21* knockout by utilizing the Cre-Lox recombination system (Figure S3A). The heterozygous *Twinkle*<sup>dup 353-365</sup> (Deletor) males and PGK-Cre females were first crossed for two generations with *Fgf21*<sup>LoxP/LoxP</sup>. The Deletor x *Fgf21*<sup>LoxP/LoxP</sup> males were then crossed with PGK-Cre x *Fgf21*<sup>LoxP/LoxP</sup> females. PGK-Cre has ubiquitous expression. All genotypes were born in the expected Mendelian ratios and were viable. An equal percentage of animals (80-86%) in all study groups survived until euthanizing at 22-24 months of age. The population was maintained with crosses between Deletor-FKO males and FK0 females. Male mice were used for all analyses because of slightly more pronounced findings of mitochondrial myopathy in Deletor males than females.

#### Cell Lines

The human fibroblasts cell line was established from a healthy 45-years-old male Caucasian donor via skin biopsy using routine protocols. Primary low passaged human myoblasts were from a healthy voluntary 45-years-old male Caucasian donor (Ahola et al., 2016). Murine C2C12 myoblasts were obtained from ATCC.

#### MEF isolation

Embryonic fibroblasts were cultured from E13.5 WT and FGF21KO mouse embryos. Embryos were collected in phosphate-buffered saline (1xPBS, without calcium and magnesium ions) and the head and liver were separated. Remaining tissue material was cut into small pieces and homogenized in 5% trypsin in 1xPBS (1ml/embryo). This suspension was incubated at +37°C and mixed by pipetting every 5 minutes. 1ml of this suspension was added to 9 ml of DMEM media supplemented with GlutaMax (Gibco), 10% FBS, amino acids (MEM Non-Essential Amino Acids Solution, Thermo Fisher) and penicillin/streptomycin. Cells were plated on gelatin-coated dishes. Confluent dishes were split the following day and harvested on day 3-4 by trypsinization for further analyses.

#### Cell culture

Human myoblasts from a healthy 45-years-old male Caucasian donor (Khan et al., 2017) were grown in 420 ml Ham's F10 with GlutaMax (Gibco 41550-021) supplemented with 75 ml of heat-inactivated

fetal bovine serum (FBS, Sigma, F9665), 250 mg bovine serum albumin (BSA, Sigma #A-4503), 250 mg Fetuin (Sigma #F-2379), 90 mg insulin (Sigma #I-1882), 5 ml of 39 µg/ml dexamethasone (Sigma, #D1756), 500 µl epidermal growth factor (10 µg/ml, BD Biosciences, 354052), 10 mg uridine (Calbiochem, 6680). All other cells were grown in Dulbecco's Modified Eagle's Medium DMEM (Sigma-Aldrich), with 4.5 g/l glucose supplemented with 10% fetal bovine serum, 20 mM pyruvate, 100 units/ml of penicillin/streptomycin and 50 µM uridine. All cells were maintained in a humidified chamber at 37°C at 5% CO<sub>2</sub>

### Respiratory function testing in animals

Oxygen consumption and carbon dioxide production were measured in Oxymax Lab Animal Monitoring System (CLAMS; Columbus Instruments). In CLAMS the mice were housed in individual cages in temperature-regulated chamber, settled for 24 hours and were recorded for 2 days. The settling and the first day of recording were in room temperature (+22°C) followed by 24 hours at thermoneutral conditions (+30°C). Respiratory exchange ratio (RER) was calculated to indicate a rough estimate of the preferred fuel (carbohydrate breakdown vs. fatty acid oxidation) of the organism.

### Tracer assays

#### *Pre-conditioning:*

For fasting experiments food was removed after initial feeding period at 7-9 pm (beginning of dark period) and tracer uptake took place in the following morning after 24 hours of fasting.

For FGF21 injection experiments, animals were intraperitoneally injected 4 hours prior to tracer injection with either saline placebo or recombinant mouse FGF21 (see details for production in method details). The injected dose for recombinant FGF21 was 0.06 mg/kg.

Rapamycin treatment was made according to (Khan et al., 2017). Rapamycin (LC Laboratories #R-5000) was dissolved in DMSO to yield stock of 100 mg/ml, further diluted in 5% PEG-400 to final concentration of 1.2 mg/ml. Solution was sterile filtered and stored at -80°C prior to use. Before administration, mouse was weighed and the dose was adjusted to 8 mg/kg/day. Mice were injected intraperitoneally for 6 weeks.

#### *Assay protocol:*

Prior to all tracer uptake assays, food was removed from cages for 3 hours. The animals were kept in anesthesia with small dose of inhaled isoflurane (1-1.5% in oxygen or room air carrier) on temperature-controlled mats throughout the procedure. Glucose analog <sup>18</sup>F-FDG (<sup>18</sup>F-Fludeoxyglucose; total activity injected 5MBq) or fatty acid analog <sup>18</sup>F-FTHA (<sup>18</sup>F-Fluoro-6-thia-heptadecanoic acid; total activity 5MBq) were administered intravenously. The uptake periods for <sup>18</sup>F-FDG and <sup>18</sup>F-FTHA were 60 and 30 minutes, respectively. Biodistribution of tracers was assayed by detecting gamma rays emitted from excised tissues with manual gamma counter. Standardized uptake values (SUV) in different tissues were calculated for each sample. Quantitative phosphor imaging autoradiography of <sup>18</sup>F-FDG in brain was performed after 60 minutes of *in vivo* uptake period. Coronal brain cryosections (20 µm) were cut on objective glass and exposed on imaging plate (plate (Fuji BAS-TR2025) for approximately 3.5 hours. The plates were scanned with Fuji BAS-5000 Phosphor imager with 25 µm resolution (digital autoradiography, ARG). The ARG images were analyzed for count densities (photostimulated luminescence per unit area, PSL/mm<sup>2</sup>) with a computerized image analysis program (AIDA image analyzer). Cerebellar cortex was selected to be the reference brain region for signal normalization as it showed equal uptake in all study groups in *ex vivo* tissue-panel assay.

## METHOD DETAILS

### Isolation of mitochondria

Mitochondria were isolated from quadriceps femoris (QF) from Deletors to enrich for mitochondrial protein. QF was homogenized in a Potter-Elvehjem with a rotor-driven pestle in HIM buffer (10 mM HEPES-KOH, pH7.6, 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10% glycerol, 0.1% fatty-acid free BSA) supplemented with 1 mM PMSF and protease inhibitors (Sigma). One QF was lysed in 5 ml HIM buffer, strained with a 100 µm cell strainer and sieve rewashed with 5 ml homogenization buffer. Enriched mitochondrial fractions were obtained by differential centrifugation at a low step at 700xg for 20 min and resulting supernatant at 10`000xg for 10 min and was repeated. All steps were performed at 4°C.

### MtDNA deletion load and copy number determination

Total cellular DNA was isolated from frozen quadriceps femoris (QF) by standard proteinase K and phenol–chloroform method. For determination mtDNA deletion load we used long-range PCR reactions for deleted and undeleted fractions of mtDNA (Ahola-Erkila et al., 2010) using primers in non-deleted region between 16S rRNA and ND1 to enable partly deleted mtDNA molecules. 10 ng of total genomic DNA was amplified with primers for long template PCR using Phusion high-fidelity DNA polymerase (Thermo Fisher) and supplied GC buffer PCR reaction was initial denaturation for 30 s at 98°C, 22 cycles of 10 s at 98°C and 12 min extension at 72°C and a final extension step for 10 min at 72°C. For total mtDNA amount determination a non-deleted mtDNA fragment was amplified using Phusion high fidelity DNA polymerase and GC buffer: initial denaturation of 30 s at 98°C; 21 cycles of 10 s at 98°C and 2 min at 72°C; final extension step of 10 min at 72°C. All PCR products were agarose gel (1%) electrophoresed and imaged using ChemiDoc™ XRS+ System (Bio-Rad). Amount of the total mtDNA product was compared to the deleted mtDNA products.

Alternatively, mtDNA deletion load was determined by a modified a triplex assay (Rygiel et al., 2015) adapted for mice. The mouse triplex assay was used for single-well detection using 5'-modified primers with ROX (D-Loop probe), HEX (ND1 probe) or FAM (ND4 probe) for single-well detection. Primer efficiency and linear range of amplification was determined by serial dilution. A standard curve was included in every run. All primer sequences can be found from Table S6.

### Gene expression analysis

RNA was extracted by standard Trizol-chloroform precipitation for tissues and by using the Qiagen miRNA kit for cells. DNase digestion was performed and total RNA reverse transcribed using the Maxima first strand RT (ThermoFisher). 10-20 mg of tissue were used for homogenization. Total cellular RNA was extracted from snap frozen tissues in TRIzol reagent (Invitrogen) and homogenized with Fast-Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). RNA of cultured cells was directly lysed in TRIzol and extracted. RNA concentration was measured with NanoDrop1000 Spectrophotometer and integrity checked in agarose gel electrophoresis. Two micrograms of RNA was DNase treated (Ambion). One microgram of total RNA was used to generate cDNA using Maxima first-strand cDNA synthesis kit (Thermo Scientific). Quantitative real-time PCR amplification of cDNA was performed with IQ SybrGreen kit (Bio-Rad) on CFX96 Touch qPCR system (Bio-Rad). Relative expressions were normalized to β-actin or 18S rRNA as indicated. All primer sequences can be found from Table S6.

### Gene silencing

Gene silencing was performed using SilencerSelect (ThermoFisher) siRNAs for control siRNA 1 (s4390843) & 2 (s4390846) and against ATF4 (s1703, s1704), ATF3 (s1699, s1701), ATF5 (s22424, s22425). Briefly, two consecutive rounds of 25 picomoles of siRNAs per 6 cm<sup>2</sup> dish were used to transfect human fibroblasts on day 1 at 50% confluency and day 4 in order to achieve maximum

knockdown efficiency using Polyplus jetPRIME transfection kit. Transfection efficiency was confirmed by RT-qPCR.

### Western Blotting

Total protein from cells was extracted using RIPA buffer supplemented with phosphatase inhibitors (ThermoFisher Scientific) and sodium orthovanadate in order to preserve phosphorylation sites. Briefly, tissues (20 mg) were homogenized in 20-30 volumes of 1XTBS with bead homogenizer (5000 beats for 20s) (Precellys) and Triton-X was added to yield 1% volume of the extract. The mixture was incubated on ice for 30 min and collected by centrifugation at 14,000xg for 20 min. Protein concentrations were determined using Bradford assay. Proteins were separated in appropriate gels by SDS-PAGE usually on 4-20% gradient gels, transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% milk or 3% BSA in 1xTBS-T (Tris Buffered Saline with 0.1% Tween20) for 1 h and primary antibodies incubated overnight at 4°C, Ab concentration range according to manufacturer's instructions (1:250-1:1000) in 3% BSA in TBS-T buffer. Antibodies used are listed in key resources table. Secondary HRP antibodies were used in 1:10000 dilution in 5% milk in TBS-T and signal detected by Clarity Western ECL Substrate (Biorad) with ChemiDoc™ XRS+ System (Biorad). Signal intensities were quantified by ImageLab 5.2.1 (Biorad).

### Histology

*Respiratory chain enzyme activity and Oil-red-O, fresh-frozen tissues.* Tissues were freshly embedded in OCT medium (Tissue-Tek) and frozen in 2-methylbutane bath under liquid nitrogen cooling.

Simultaneous histochemical activity analysis of cytochrome-c-oxidase (COX) and succinate dehydrogenase (SDH) activities was performed on QF and brain slices (12 µm), incubation times being 30 minutes for COX (RT) and 40 minutes for SDH (+37°C). Buffer contents for COX: 0.05 M phosphate buffer (pH 7.4) with 3, 3 –diaminobenzidine (DAB), catalase, cytochrome c and sucrose and for SDH: 0.05 M phosphate buffer (pH 7.4) with nitro-blue tetrazolium and sodium succinate. After stainings the slices were dehydrated in ascending alcohols, xylene treated and mounted. From muscle sections, COX-negative/SDH-negative, COX-negative/SDH-positive and normal fibers were quantified.

For Oil-Red-O staining 8 µm sections of frozen liver were fixed in formic calcium and incubated in Oil-Red-O solution for 10 min followed by brief hematoxylin staining for nuclei.

*Immunohistochemistry in tissues:* Tissues were fixed in 10% buffered formalin and embedded in paraffin. The samples were blocked for non-specific staining by incubating for 30 min in 2% horse serum. Citric acid antigen retrieval and H<sub>2</sub>O<sub>2</sub> treatment were performed before blocking in room temperature and primary antibody incubation for overnight at 4°C. Detection was done with Vectastain Elite ABC HRP Kit according to the manufacturer's instructions followed by chromogen DAB staining. The nuclei were briefly counterstained with hematoxylin. Antibodies: SDHA (Complex II) Abcam, ab14715 (1:500); UCP1 Abcam, ab10983 (1:500).

*Immunofluorescence in tissues:* Tissues were fixed in 10% buffered formalin and embedded in paraffin. Citric acid antigen retrieval was performed. The samples were blocked for non-specific staining by incubating for 30 min in 2% horse serum. Incubation with antibodies was done in Antibody diluent (Dako), primary antibody incubation for overnight at 4°C and secondary in room temperature with appropriate Alexa Fluor® antibodies (from Abcam). Antibodies: GFAP from Millipore, AB5804 (1:500); IBA1 from Wako, 019-19741 (1:1000); MAP2 from Abcam, ab5392 (1:500) and TOM20 from Santa Cruz, sc-11415 (1:500).

*Adipocytes:* Formalin-fixed paraffin-embedded sections were stained with hematoxylin and eosin.

*Staining in cells:* Living cells grown on cover slips were stained for 15 min in 50 nM Mitotracker CMXRos in full media, rinsed with 1xPBS and fixed in ice-cold acetone or directly fixed in ice-cold acetone for immunofluorescence stainings. Fixed samples were blocked (5% horse serum, 1% BSA, 0.1% Triton-X-100 in 1xPBS) and incubated with the primary antibodies against TOM20 (SantaCruz, #11415, 1:250) and ATF4 (SantaCruz B-3, sc390063) overnight. Secondary anti-rabbit Alexa488 was used for TOM20 and anti-mouse Alexa594 for ATF4. All samples were mounted in VectaShield (Vector Laboratories) containing DAPI to counterstain for nuclei. Images were recorded on a Zeiss AxioImager 1.2 equipped with an ApoTome.

*Electron microscopy:* attached cells were directly fixed in 2% glutaraldehyde in phosphate buffer, post-fixed in 1% osmium tetroxide and dehydrated through ascending concentrations of alcohol and embedded in Epon812. 60nm ultrathin sections were obtained on a Reichert-Jung Ultracut ultramicrotome equipped with Diatome (Switzerland) diamond knife, transferred to copper grids, stained with uranyl acetate and lead citrate and observed in a Philips CM12 electron microscope at 80kV. Images were recorded with a Morada digital camera and analyzed using iTEM software.

### **Quantification of FGF21**

Terminal blood samples were collected via heart puncture, were let to coagulate for 15 minutes at room temperature and centrifuged at 4°C at 3,000x g for 15 minutes. Separated serum was frozen and stored in -80°C prior to assay. Mouse/Rat FGF21 Quasitine ELISA (R&D Systems) was used according to manufacturer's instructions. Absorbance measurement was made with SpectraMax 190 absorbance microtiter plate reader.

### **Metabolomics**

Metabolomic analyses were performed from 20 mg of muscle extract using Waters Acquity ultra performance liquid chromatography (UPLC) and triple-quadrupole mass spectrometry analysis in FIMM (Institute for Molecular Medicine Finland). All metabolite standards, ammonium formate, ammonium acetate and ammonium hydroxide were obtained from Sigma-Aldrich (Helsinki, Finland). Formic acid (FA), 2-propanol, acetonitrile (ACN), and methanol (all HiPerSolv CHROMANORM, HPLC grade, BDH prolabo) were purchased from VWR International (Helsinki, Finland). Isotopically labelled internal standards were purchased from Cambridge Isotope Laboratory. The exact procedure is described and validated in (Nandania et al., 2018a; Nandania et al., 2018b). The polar metabolome was extracted from frozen 20 mg mouse muscle and lysed with a Precellys homogenizer in 1.4 mm ceramic (Zirconium oxide) beads containing tubes including 10  $\mu$ l of labeled internal standard mix and 1:20 sample: extraction solvent in a two-step extraction protocol. The first step contained 10 parts of 100% ACN + 1% FA for 3 cycles at 5500 rpm with 30s pausing in-between. Sample was centrifuged at -20°C at 2 min in an Eppendorf 5404R centrifuge at 5000 rpm and supernatant collected. The remaining pellet was re-lysed with 10 parts of 80/20%ACN/H<sub>2</sub>O + 1% FA as described above. Calibration solutions were serially diluted in a 96-well plate. Samples were analyzed on an ACQUITY UPLC-MS/MS system (Waters Corporation, Milford, MA, USA) with specific settings reported in (Nandania et al., 2018b). Detection was performed on a Xevo® TQ-S tandem triple quadrupole mass spectrometer (Waters, Milford, MA, USA), was operated in both positive and negative polarity. Samples were ionized by electro spray ionization (ESI) and dwell time and subsequent data acquisition were automatically calculated by MassLynx 4.1 software and processed using TargetLynx software. Quantification was performed by internal standards and external calibration curves.

### dNTP pools

Total dNTP pool measurements were performed and analyzed as described (Marti et al., 2012; Nikkanen et al., 2016). Approximately 50-100 mg of frozen muscle tissue was homogenized in 10 volumes (v/w) homogenization buffer (10 mM Tris/HCl, pH 7.5, 0.2 mM ethylene glycol tetra-acetic acid (EGTA), 0.5 % bovine serum albumin (BSA), 210 mM mannitol, 70 mM sucrose) using a polytron homogenizer. Obtained homogenate was centrifuged for 10 min at 4°C. Cold methanol (-20°C) was added to the resulting supernatants for a final concentration of 60% (v/v) and stored at -80°C for 3h. Samples were then heated to 95°C for 3 min, cooled on ice and centrifuged at 16000 x g at 4°C for 20 min. Obtained supernatants were collected and dried down. Appropriate amounts of re-hydrated extracts were added to the reaction mix containing 40 mM Tris/HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol (DTT) and 0.25 μm specific primed oligonucleotide, 0.75 μm <sup>3</sup>H-dTTP or <sup>3</sup>H-dATP, 0.30-unit Taq DNA polymerase for a total of 20 μl and incubated for 60 min at 48°C. 15 μl of reaction mix was spotted onto DE-81 filter paper and dried. Spotted samples were then washed 3x with 5% Na<sub>2</sub>HPO<sub>4</sub>, 1x water and 1x with 95% ethanol. Reaction products were analyzed using a liquid scintillation counter with 3-6 measurements per individual sample and quantified compared to standard curves.

### RNA sequencing of patient muscle

A Bergström needle biopsy from *vastus lateralis* was collected from eight controls and from four PEO patients. Total muscle RNA was extracted with standard TRIzol and chloroform method and purified with RNA purification kit (RNeasy; Qiagen). A total of one microgram of total RNA was used for global transcriptomics analysis performed by the Beijing Genomic Institute (BGI) using their standard protocols. In brief, transcriptome sequencing was performed using 50bp paired-end sequencing on an Illumina sequencing platform. The resulting raw reads were preprocessed by the sequencing provider to filter out reads failing criteria for quality, unknown base and adaptor sequence content. Clean reads, specified in FASTQ format, amounted to approximately 26 million reads per sample. Subsequent analysis was performed in-house and reads were aligned against the GRCh38 Human genome assembly (DNA primary assembly in FASTA format and gene annotation in GTF format from Ensembl release 92) using STAR 2.5.0a, giving read alignments in BAM format. Counting of reads mapping to genes was performed with htseq-count, HTSeq 0.10.0, in union overlap resolution mode. The obtained read counts were then used for differential gene expression analysis with the Bioconductor DESeq2 1.18.1 package. Fold changes in binary logarithmic scale and respective p-values were extracted for pairwise comparisons.

### Production of recombinant mouse FGF21

*Cloning and expression:* Champion pET SUMO Protein Expression system was used (Invitrogen). BL21(DE3) *E. coli* cells with pET-SUMO-FGF21 expression vector were grown at +32°C in Luria-Bertani media until the OD600 was 0.6-0.8. Then, 0.3 mM of isopropyl-D-thiogalactoside (IPTG) was added to induce expression of the fusion protein and cells were grown for another 4 hours. Cell cultures were pelleted at 8000 rpm for 10 min at +4°C, washed with 25mM Tris-HCl, pH7.5, 200mM NaCl, centrifuged for 15 min at 5000rpm, +4°C and frozen at -20°C.

*Purification:* Whole purification procedure was performed at +4°C. All chromatography steps were done on AktaPurifier 10 (GE Healthcare). A cell pellet harvested from 1 L of culture was melted, suspended in 50ml of 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH7.6, 150 mM NaCl, 0.5mM PMSF and disrupted by sonication. Cell lysate was ultra-centrifuged at 40,000 rpm for 30 min and supernatant was collected. NaCl and imidazole were added to yield final concentrations of 500 mM and 20mM, respectively. Protein solution was filtered through 0.45μm PVDF filter (Millipore) and loaded at 3 ml/min flow rate onto HisTrap Crude FF 5ml column (GE Healthcare) equilibrated with 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6, 500



mM NaCl containing 20 mM imidazole. After washing with 10 x column volume of 20 mM imidazole and 5 x column volume of 50 mM imidazole, the his-tagged sumo-FGF21 fusion was eluted with 200 mM imidazole in the equilibration buffer. Eluate was concentrated with 10 kDa cut-off Vivaspin concentrators (Millipore), protein was desalted against 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6 using HiTrap Desalting column 5ml (GE Healthcare) at 3 ml/min flow rate. Then the protein was loaded at 3ml/min flow rate onto HiTrap DEAE FF 5ml column (GE Healthcare), equilibrated with 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6. After washing with 120mM NaCl, the protein was eluted with 200 mM NaCl in the equilibration buffer. Pooled fractions with eluted protein were concentrated to 1ml in Vivaspin concentrators with 10kDa cut-off (Millipore) and desalted on HiTrap Desalting column 5ml (GE Healthcare) against 5 mM TrisHCl, pH 8.0, 155 mM NaCl. Cleavage of sumo tag was performed using SUMO protease (Invitrogen) according to manufacturer's instructions. After removal of NP-40 with BioBeads (Bio-Rad) followed by adjustment of NaCl concentration to 500mM and imidazole concentration to 20 mM the protein solution was loaded at 0.5 ml/min onto HisTrap FF 1 ml column (GE Healthcare) equilibrated with 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6, 500 mM NaCl, 20 mM imidazole. Flow through containing FGF21 was concentrated to 400 $\mu$ l in Vivaspin concentrators and loaded onto Superdex 75 10/300 GL column equilibrated with PBS, pH7.4. Gel filtration was run at 0.2 ml/min. Fractions containing FGF21 were pooled and concentrated. Protein concentration was determined using Bradford method with bovine serum albumin as a standard.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses and their graphical representation were performed in GraphPad Prism v.7.0 software (GraphPad Software, Inc., USA). Statistical test used in each experiment is indicated in figure legend. P-values of less than 0.05 were considered statistically significant. Outlier analysis for all analyses was done using GraphPad Prism 6.0 ROUT method (Q=1%) and false positive analysis for metabolomics with Benjamin-Hochberg method, critical value of 0.2.

## DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and its supplementary information files. Materials used in this study are available from the corresponding author on reasonable request. Human muscle biopsy RNA-Seq data deposited to Gene Expression Omnibus data repository with accession number GSE129811.

## Supplementary data title and legends

### **Figure S1, related to Figure 1. UPR<sup>er</sup> markers in skeletal muscle**

**(A)** Endoplasmic reticulum stress -related unfolded protein response (UPR<sup>er</sup>) related protein markers. BiP (binding immunoglobulin protein) in the muscle of 22-month-old Deletor mice. Positive control: tunicamycin (TUN) exposure in cultured mouse myoblasts (C2C12). Western blot analysis, WT and Deletor n=4 per group.

**(B)** X-box binding protein 1 transcript analysis. *Xbp1* gets spliced upon endoplasmic reticulum stress, but is intact in Deletor muscle. PCR of cDNA and gel electrophoresis. Young Deletors and WT n=2 per group, aged Deletors n=5 per group.

**(C)** Expression of *Atf6*, a regulator of ER-stress response, is not induced in the muscle of 24-month-old Deletor mice. WT n=5 and Deletor n=9, qPCR analysis. Bars represent the group average with SD. Individual animals are shown as dots.

### **Figure S2, related to Figure 2. Morphology changes of mitochondria upon mitochondrial translation stalling in myoblasts.**

Mouse and human cultured myoblasts (MB). Staining with Mitotracker (red) and DAPI=4',6-diamidino-2-phenylindole (DNA binding dye); lowest panel: transmission electron microscopy (TEM). Actinonin exposure for 6h and 24h.

### **Figure S3, related to Figure 3. Characterization of Deletor-FGF21KO**

**(A)** Crossing strategy to produce DEL-FKO and FKO animals. Twinkle mutation always inherited from the male.

**(B)** Validation of FGF21 knockout efficiency. 24-hr fasting effect on serum FGF21 levels in five-month-old WT and FGF21KO mice, indicating the lack of FGF21 response in FGF21 knockouts; n=2 per group.

**(C)** Age-effect on serum-FGF21 concentrations in Deletors of different ages. Increase in FGF21 is evident after 16-20 months of age. WT n=6, Deletors n=4 per age group.

**(D)** Whole body metabolism analysis; metabolic cages. Carbon dioxide production and oxygen consumption recorded for 24h in room temperature and 24h in thermoneutrality (+30°C). WT, Deletor and DEL-FKO n=3 per group, FKO n=6. RER ~1: carbohydrate fuel preference, RER below 0.8: lipid preference. Light=inactivity. Dark= active period.

**(E-F)** OXPHOS complex II, III, IV and V amounts. E) Western blot detection; F) relative quantitation against mitochondrial mass marker porin. n=3 per group.

Animals were 22-24 months old if not otherwise indicated. Bars represent the group average with SD. Box: 25th and 75th percentiles with median and range. Dots indicate data points from individual animals. Pairwise two-tailed t-test, statistical significance: \* p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001, \*\*\*\*p≤0.0001. Abbreviations: ATP5A=ATP synthase subunit alpha, mtCO1=Cytochrome c oxidase subunit

1, SDHA=Succinate dehydrogenase complex subunit A, UQCRC2=Cytochrome-bc1-complex subunit 2

**Figure S4, related to figure 4. Metabolic status and ISR<sup>mt</sup> in FGF21KO**

**(A)** COX/SDH histochemical activities and pS6 immunohistochemistry on consecutive frozen sections. Scale bar 50  $\mu$ m. Phosphorylated S6 protein is induced in the same fibers that are COX-SDH deficient (circled)

**(B)** ISR<sup>mt</sup> components and *Atf3-5* are expressed in MEFs as a consequence of mitochondrial translation inhibition (actinonin) independently of FGF21. Quantitative PCR analysis in WT and FGF21KO primary MEFs.

**(C)** Volcano plots of muscle metabolomes (Deletor, FKO n=6; WT, FKO n=8). Despite accumulation of acylcarnitine pool in Deletor-FKO (DEL-FKO vs. FKO and DEL-FKO vs. WT) the metabolic profile of DEL-FKO mimics WT (DEL-FKO vs. WT) rather than Deletor (DEL vs. DEL-FKO). AMP = adenosine monophosphate, Asp = aspartate, GAA = guanidinoacetic acid, Glu = glutamate, IMP = inosine monophosphate, NAD = nicotinamide adenine dinucleotide, PE = phosphoethanolamine, Thr = threonine, TNO = trimethylamine N-oxide, 5-f-THF = formyl-THF, 5-m-THF=5-methyltetrahydrofolate. Acylcarnitines: carbon chain length followed by [C]=Carnitine.

Animal ages: 22-24 months old. Volcano plots: Statistical significance determined without correction for multiple comparisons, alpha=0.05% (horizontal line y-axis). Each metabolite analyzed individually, not assuming a consistent SD. Abbreviations: ATF=Activating transcription factor, Cth=Cystathionine gamma-lyase, COX=cytochrome-c-oxidase, Gdf15=Growth/differentiation factor 15, Mthfd2=methylenetetrahydrofolate-dehydrogenase-2 (mitochondrial), Phgdh=Phosphoglycerate dehydrogenase, Psat1=Phosphoserine aminotransferase, pS6=ribosomal S6-subunit phosphorylated form, SDH=succinate dehydrogenase

**Figure S5, related to Figure 5. Glucose uptake and pathology in CA2**

**(A)** Digital autoradiography (ARG) of representative frozen sections, coronal slice coordinates visualized on the right (hematoxylin-eosin staining). Encircled area includes DHC; magnified in the lower panel, arrows point to increased PSL/mm<sup>2</sup> in DHC. N-numbers are 6-8 animals per group.

**(B)** Left: ARG of <sup>18</sup>F-FDG in brain of WT mice with intravenous recombinant FGF21 (rFGF21) injections or 24 h fasting. Arrows: DHC. Right: Serum FGF21 in animals of the experiment on the left.

**(C)** Long-range PCR analysis of mtDNA: Top: ~16 kb intact mtDNA and additional short smear-like products represent mtDNAs with multiple different deletions. Below: ~500 bp product of D-loop region, no mtDNA deletions. **(D)** Astrocyte morphology in CA2, non-reactive. GFAP immunofluorescence, scale bar 100  $\mu$ m.

**(E)** Microglial morphology in CA2, non-inflammatory. Immunofluorescence for IBA1. Arrows indicate the cells shown in greater magnification (white box). Scale bars 100  $\mu$ m.

Animals were 22-24 months old, except in (B) 5 months. Bars represent the group average with SD. Box: 25th and 75th percentiles with median and range. Dots indicate data points from individual

animals. Pairwise two-tailed t-test, statistical significance: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ . Abbreviations:  $^{18}\text{F}$ -FDG= Fluoro-deoxy-glucose, rFGF21=recombinant fibroblast growth factor 21, CA2=hippocampal CA-region 2, DHC=dorsal hippocampus, VHC=ventral hippocampus, GFAP=Glial fibrillary acidic protein, IBA1=Ionized calcium binding adaptor molecule 1, DAPI=4',6-Diamidino-2-phenylindole (DNA dye), PSL=photostimulated luminescence,  $\Delta\text{mtDNA}$ = mtDNA with deletions.

**Table S1, related to Figure 1A. Average (FC), SD and n-number presented for each gene and comparison.** Artificial units, WT average = 1. Highlighted in red: statistically significant difference ( $p < 0.05$ )

**Table S2, related to Figure 1G. Differential gene expression analysis of representative  $\text{ISR}^{\text{mt}}$ ,  $\text{UPR}^{\text{mt}}$  and ATF -genes in human mitochondrial myopathy.** Standard error (SE). Significantly changed genes are highlighted with colours.

**Table S3, related to Figure 3G AND 3H. Average (FC), SD and n-number presented.** Artificial units, WT and FKO average = 1. Parallel comparison between [WT vs. DEL] and [FKO vs. DEL-FKO] not possible due to instrument calibration between the experiments.

**Table S4, related to Figure 4E. Average (FC), SD and n-number presented with t-tests between genotypes.** Artificial units, WT average = 1. In figure stars represented p-values of DEL vs. WT and DEL-FKO vs. WT.

**Table S5, related to Figure 4F. Average (FC), SD and n-number presented with t-tests between genotypes.** Artificial units, WT average = 1. In figure stars represented p-values of DEL vs. WT and DEL-FKO vs. WT.

**Table S6, related to STAR METHODS. Human and mouse primer sequences.**

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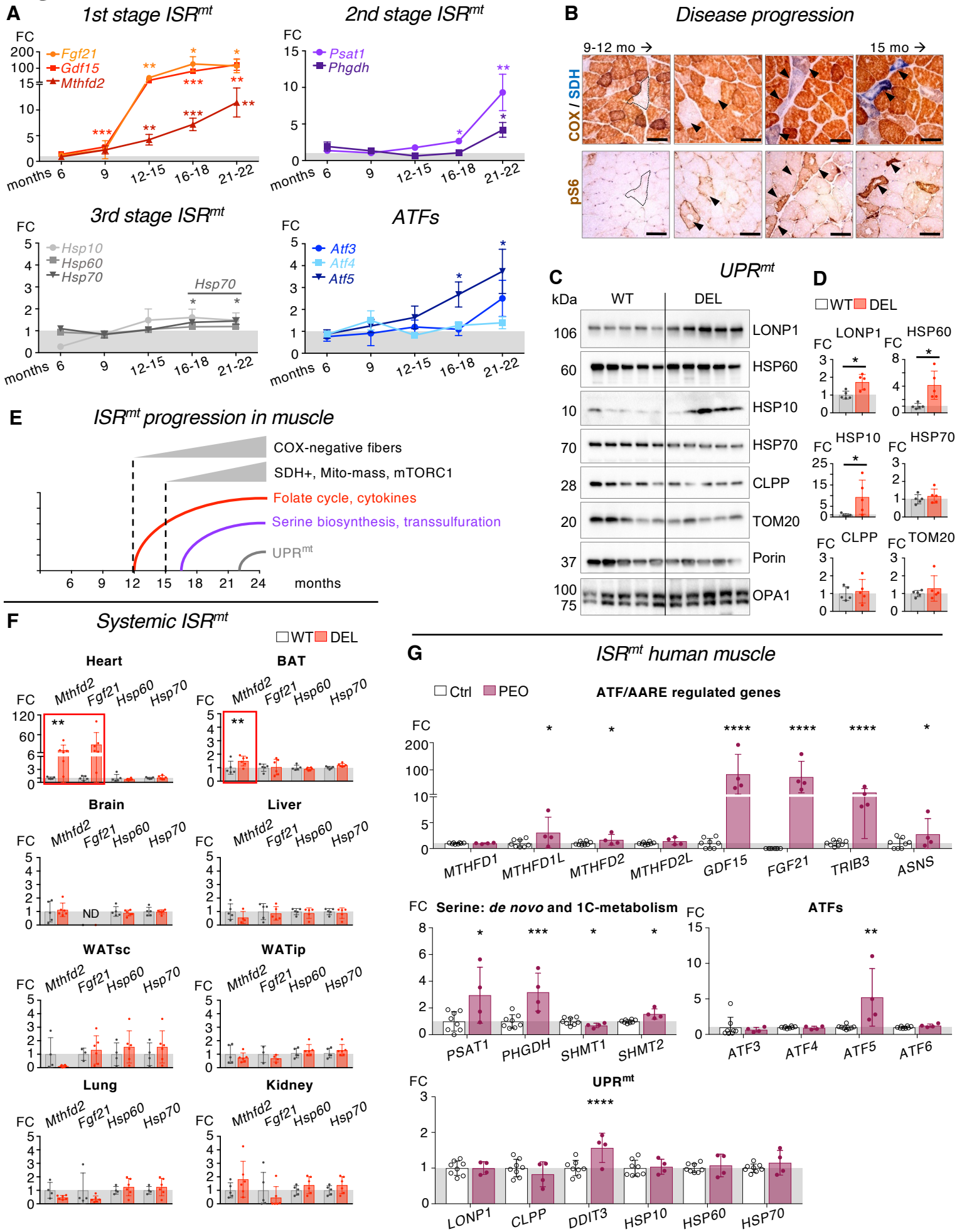
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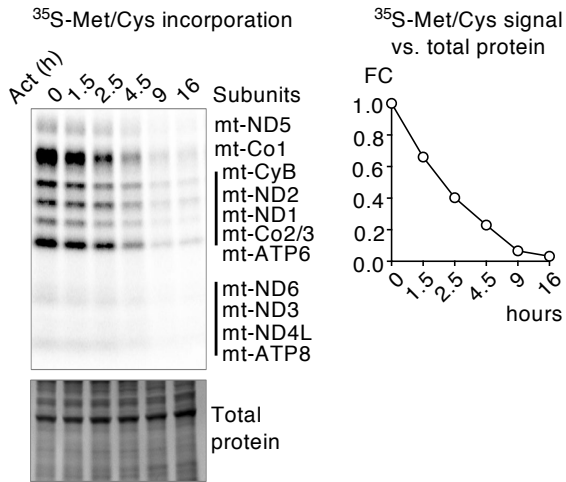
# Figure 1



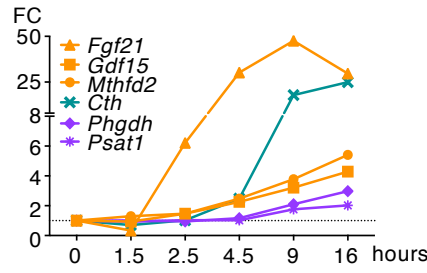
# Figure 2

## Mouse myoblasts

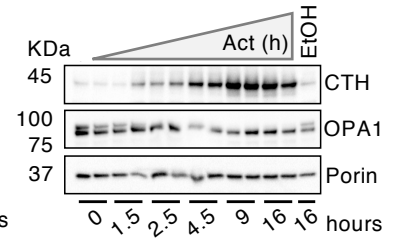
### A Mitochondrial translation inhibition



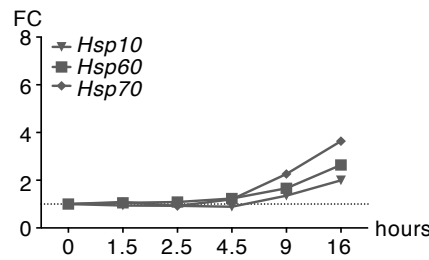
### B *ISR<sup>mt</sup>*



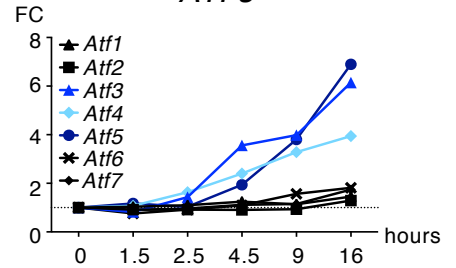
### C



### D *UPR<sup>mt</sup>*

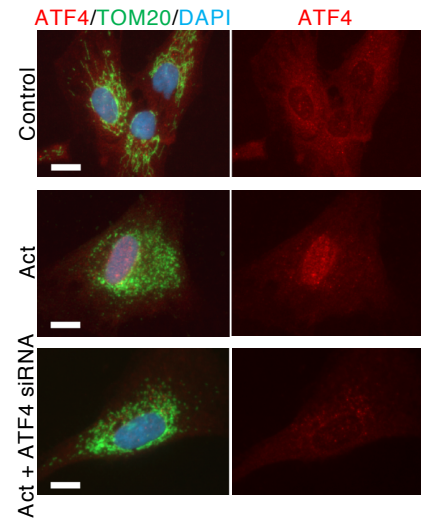


### E *ATFs*

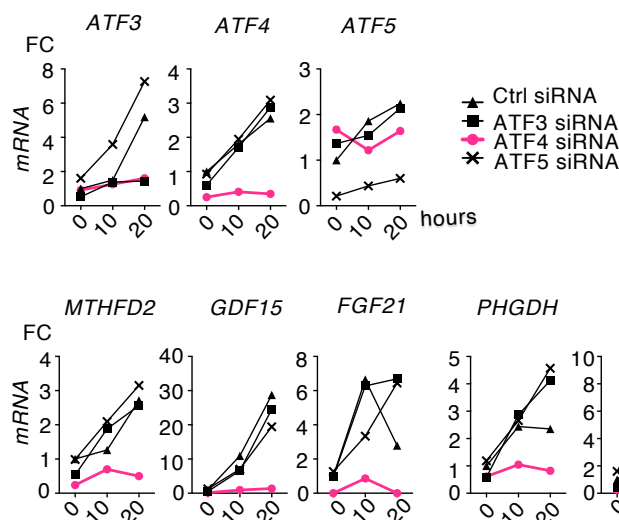


## Human fibroblasts

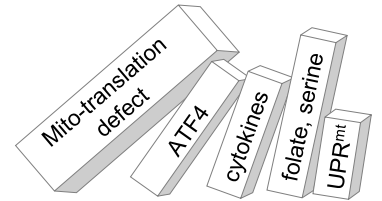
### F *ATF4* relocation



### G *ATF 3-5* inhibition

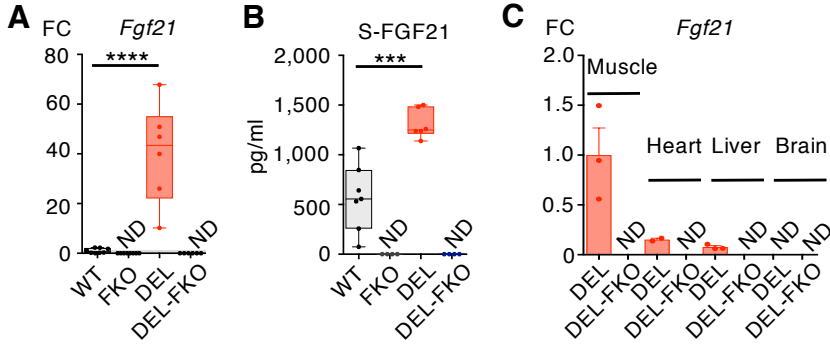


### H *ISR<sup>mt</sup>* in cultured cells

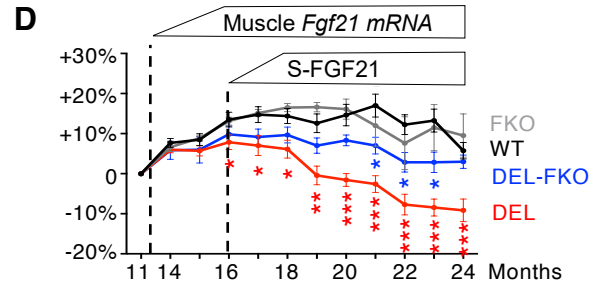


# Figure 3

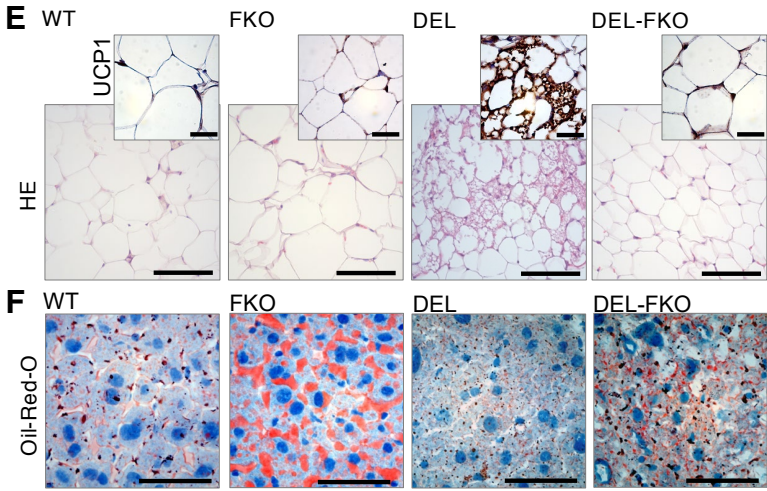
## FGF21 expression in mouse lines



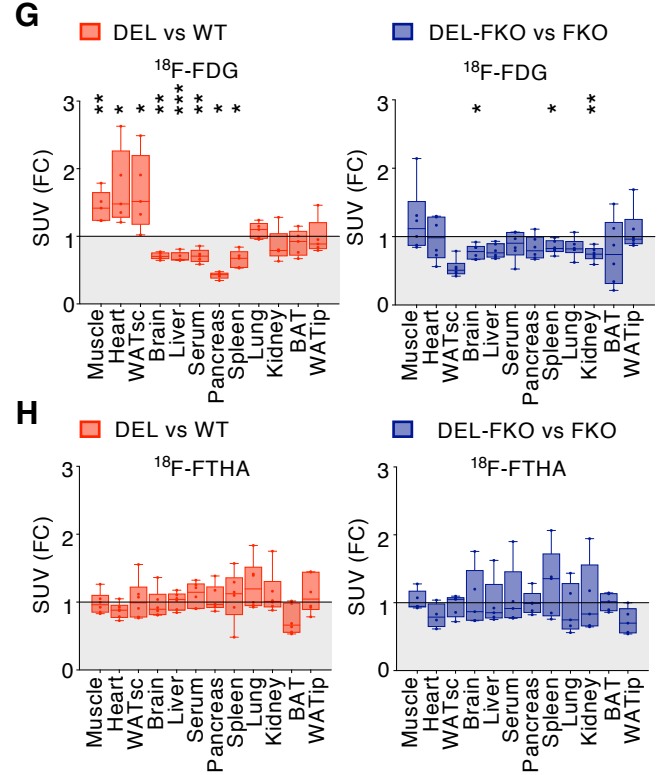
## Body weight



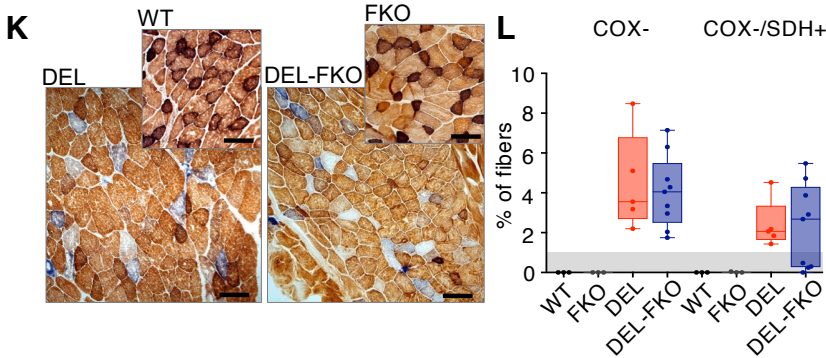
## Adiposity in mouse lines



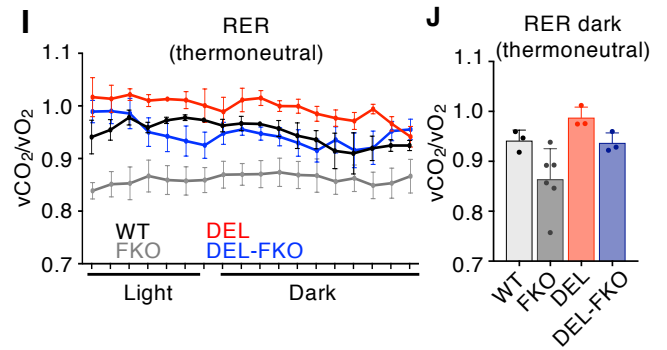
## In vivo glucose and fatty acid uptake



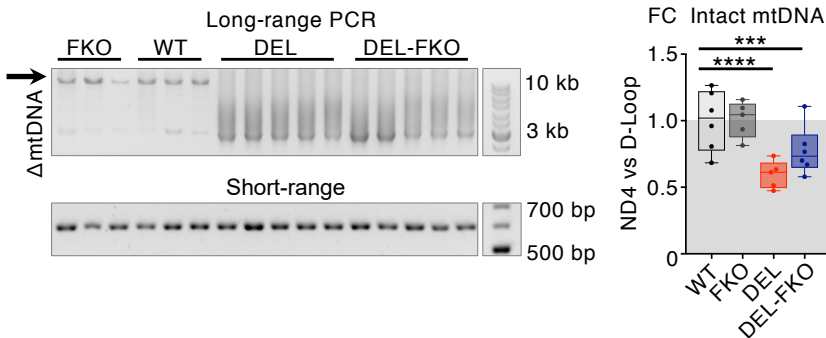
## Respiratory chain activity in skeletal muscle



## Whole body metabolism

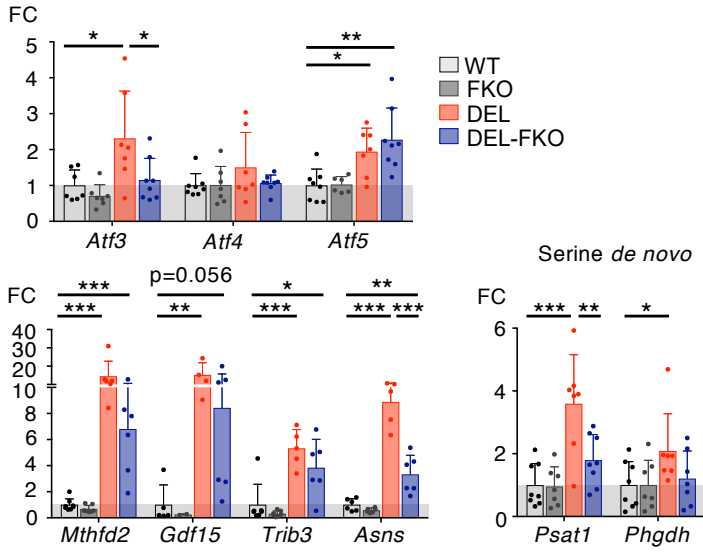


## mtDNA deletions

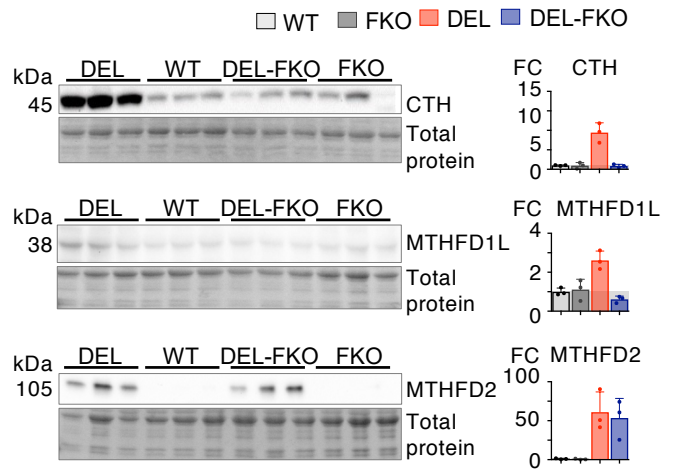


# Figure 4

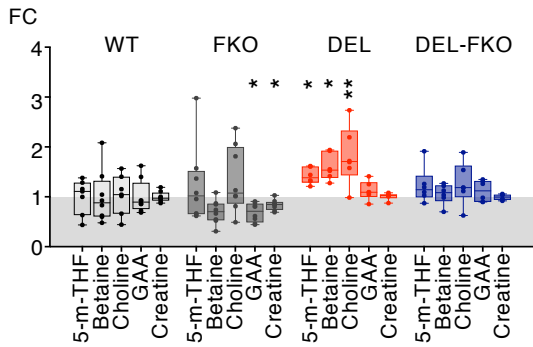
## A *ISR<sup>mt</sup> before and after FGF21-KO*



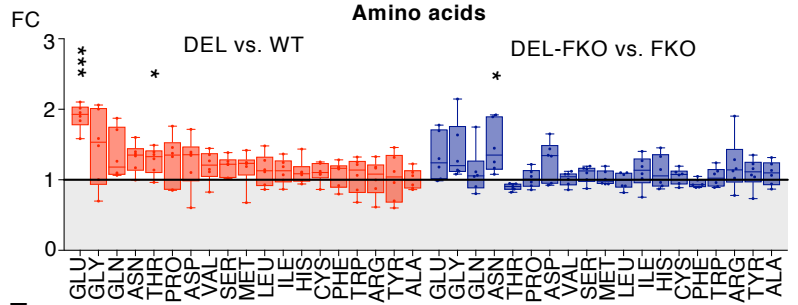
## B *Transsulfuration, Mitochondrial one-carbon cycle*



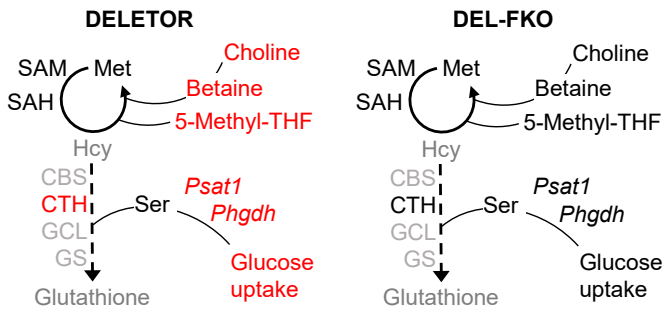
## C *Methyl cycle metabolites*



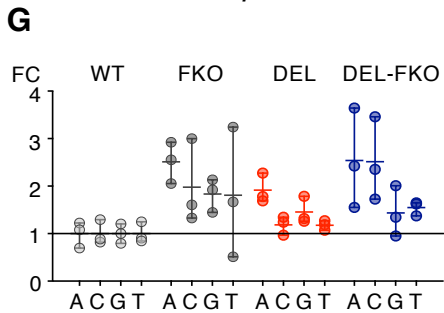
## E *Amino acid and acylcarnitine pools*



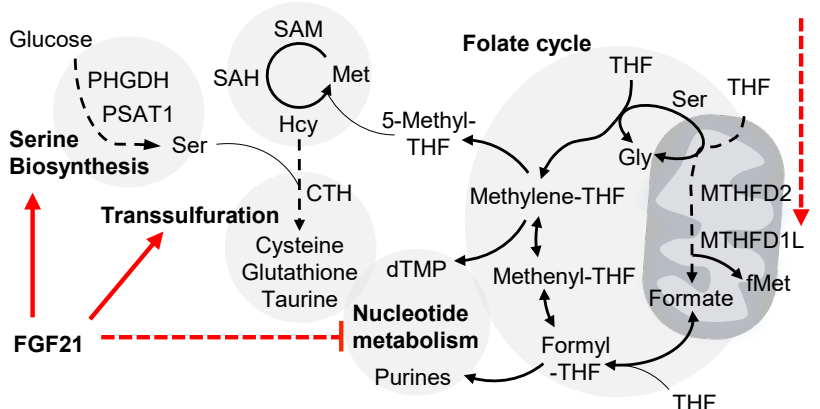
## D *FGF21 and transsulfuration pathway*



## dNTP pools

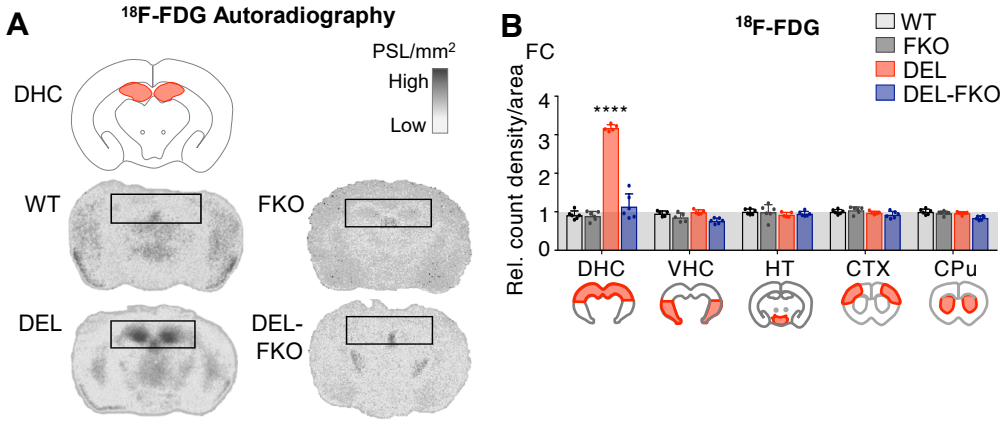


## H *One-carbon metabolism and FGF21*

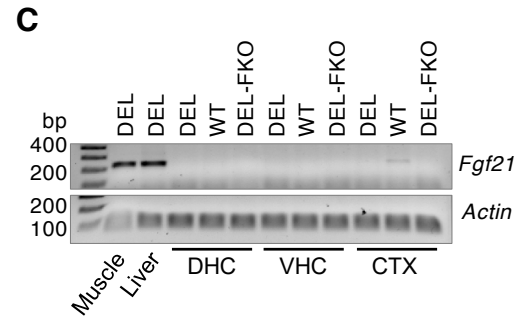


# Figure 5

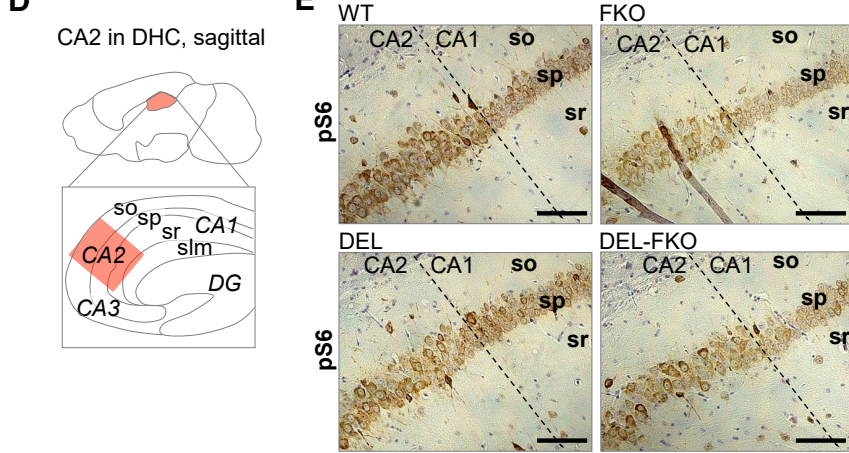
## Glucose uptake in the brain



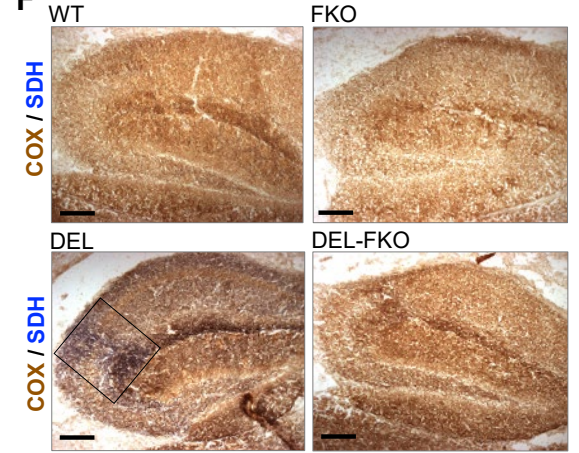
## Fgf21 expression in the brain



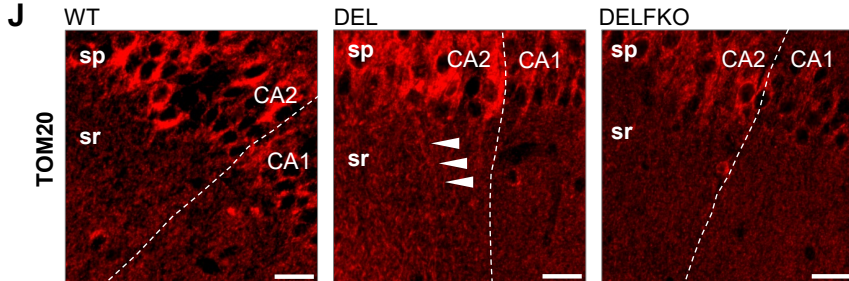
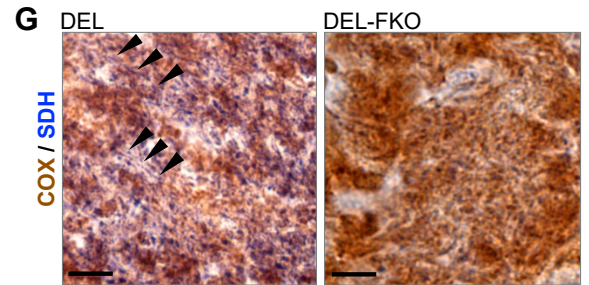
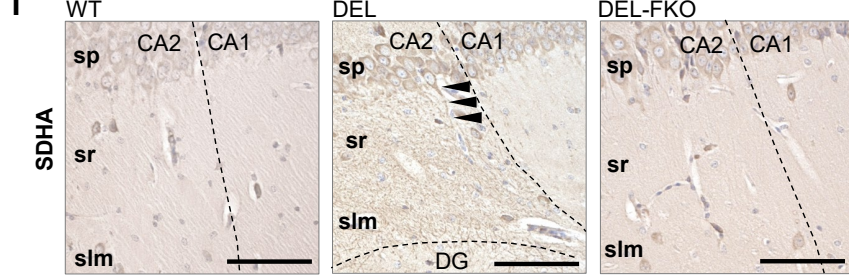
## mTORC1 activation in DHC



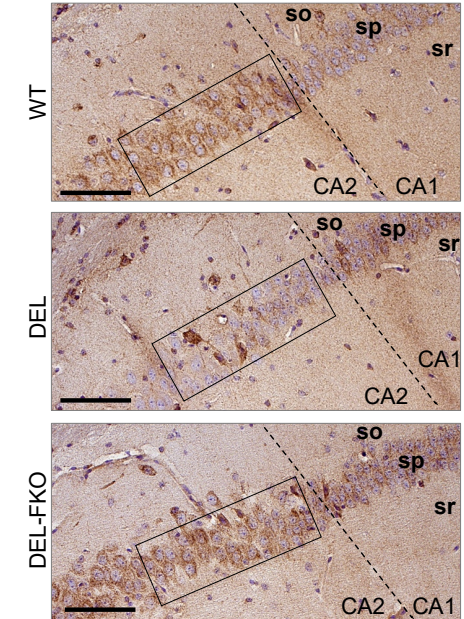
## OXPHOS in DHC



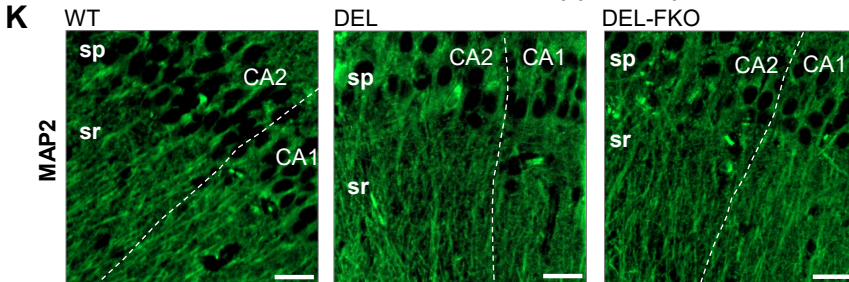
## Mitochondrial mass in DHC



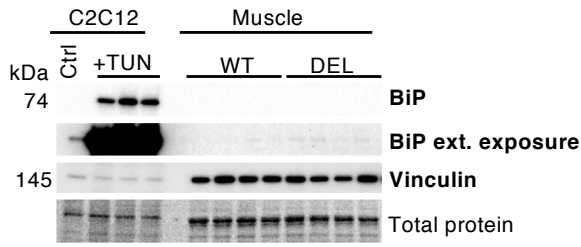
## MTCO1



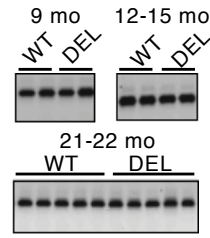
## Dendritic network in dorsal hippocampus



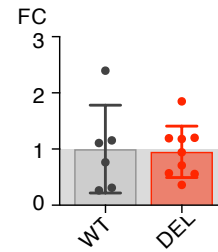
### A BiP protein levels



### B XBP1 splicing



### C *Atf6* mRNA

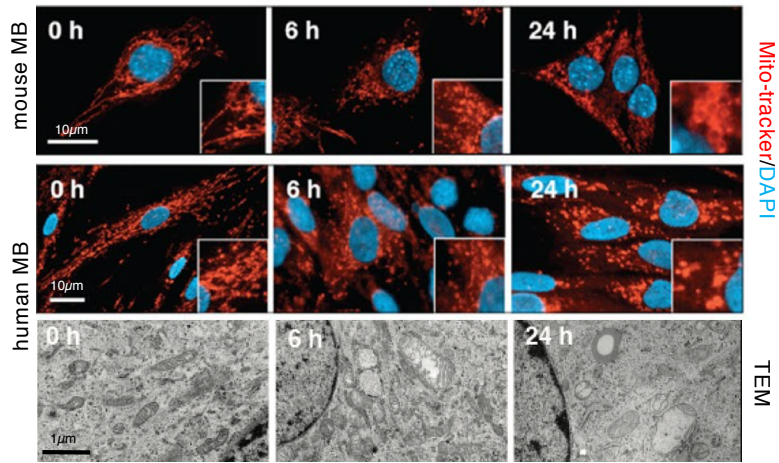


**Figure S1, related to Figure 1 and 2. UPR<sup>er</sup> markers in skeletal muscle.**

**(A)** Endoplasmic reticulum stress -related unfolded protein response (UPR<sup>er</sup>) related protein markers. BiP (binding immunoglobulin protein) in the muscle of 22-month-old Deletor mice. Positive control: tunicamycin (TUN) exposure in cultured mouse myoblasts (C2C12). Western blot analysis, WT and Deletor n=4 per group.

**(B)** X-box binding protein 1 transcript analysis. *Xbp1* gets spliced upon endoplasmic reticulum stress, but is intact in Deletor muscle. PCR of cDNA and gel electrophoresis. Young Deletors and WT n=2 per group, aged Deletors n=5 per group.

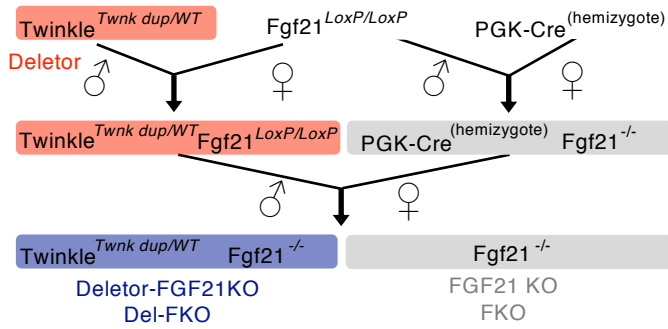
**(C)** Expression of *Atf6*, a regulator of ER-stress response, is not induced in the muscle of 24-month-old Deletor mice. WT n=5 and Deletor n=9, qPCR analysis. Bars represent the group average with SD. Individual animals are shown as dots.



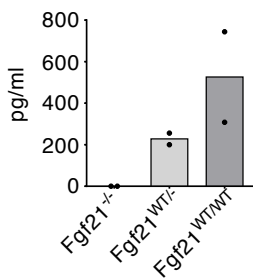
**Figure S2, related to Figure 2. Morphology changes of mitochondria upon mitochondrial translation stalling in myoblasts.**

Mouse and human cultured myoblasts (MB). Staining with Mitotracker (red) and DAPI=4',6-diamidino-2-phenylindole (DNA binding dye); lowest panel: transmission electron microscopy (TEM). Actinonin exposure for 6h and 24h.

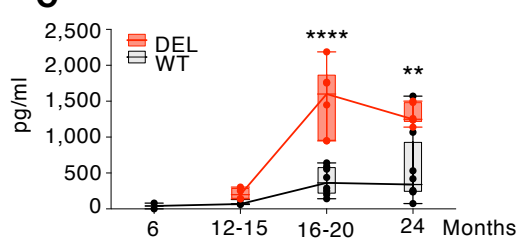
### A Deletor - FGF21KO mouse design



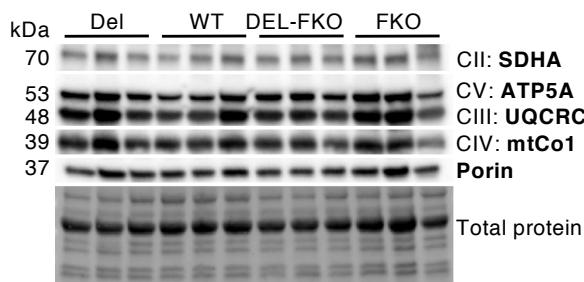
### B S-FGF21 in FGF21KO



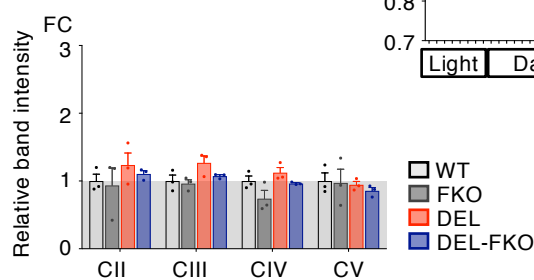
### C S-FGF21 by age



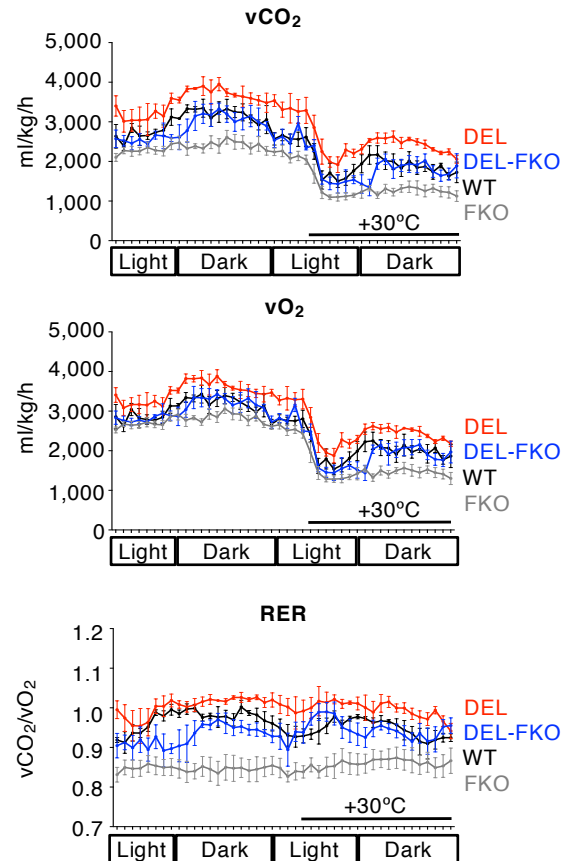
### E Respiratory chain proteins in the muscle



### F



### D Whole-body metabolism

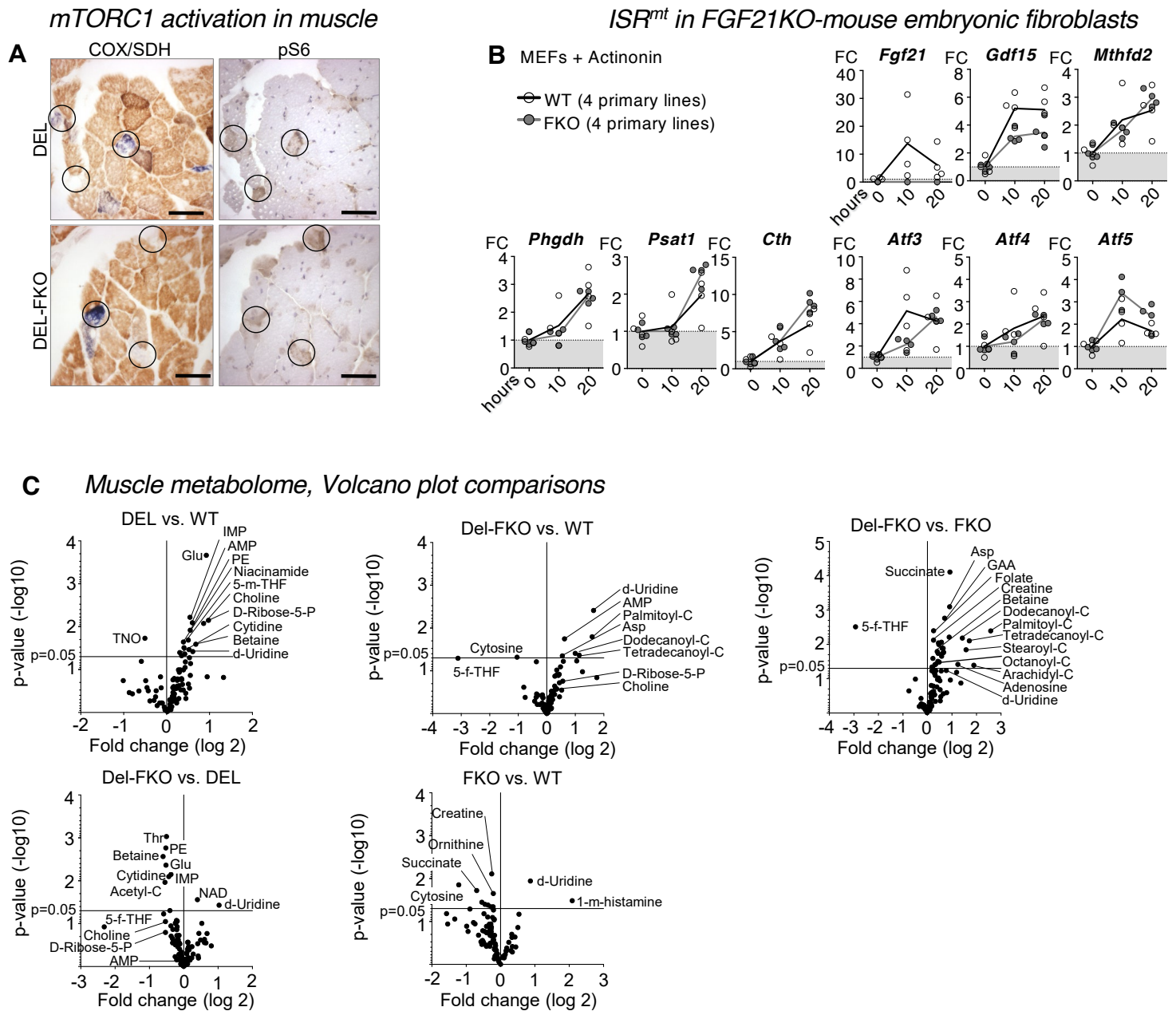


**Figure S3, related to Figure 3. Characterization of Deletor-FGF21KO.**

- (A) Crossing strategy to produce DEL-FKO and FKO animals. Twinkle mutation always inherited from the male.  
 (B) Validation of FGF21 knockout efficiency. 24-hr fasting effect on serum FGF21 levels in five-month-old WT and FGF21KO mice, indicating the lack of FGF21 response in FGF21 knockouts; n=2 per group.  
 (C) Age-effect on serum-FGF21 concentrations in Deletors of different ages. Increase in FGF21 is evident after 16-20 months of age. WT n=6, Deletors n=4 per age group.  
 (D) Whole body metabolism analysis; metabolic cages. Carbon dioxide production and oxygen consumption recorded for 24h in room temperature and 24h in thermoneutrality (+30°C). WT, Deletor and Del-FKO n=3 per group, FKO n=6. RER ~1: carbohydrate fuel preference, RER below 0.8: lipid preference. Light=inactivity. Dark=active period.  
 (E-F) OXPHOS complex II, III, IV and V amounts. E) Western blot detection; F) relative quantitation against mitochondrial mass marker porin. n=3 per group.

Animals were 22-24 months old if not otherwise indicated. Bars represent the group average with SD. Box: 25th and 75th percentiles with median and range. Dots indicate data points from individual animals. Pairwise two-tailed t-test, statistical significance: \* p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001, \*\*\*\*p ≤0.0001. Abbreviations: ATP5A=ATP synthase subunit alpha, mtCO1=Cytochrome c oxidase subunit 1, SDHA=Succinate dehydrogenase complex subunit A, UQCRC2=Cytochrome-bc1-complex subunit 2





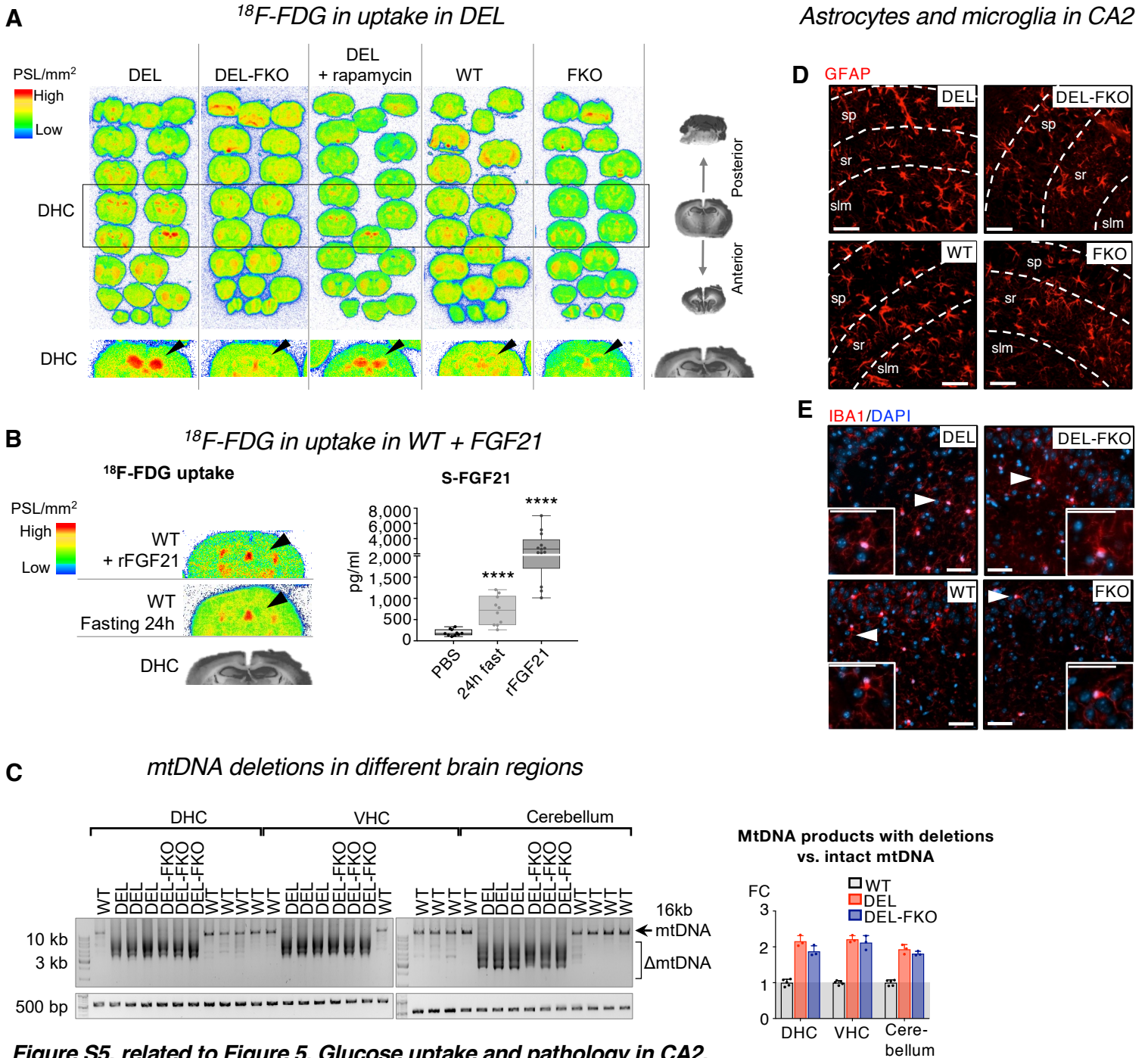
**Figure S4, related to figure 4. Metabolic status and ISR<sup>mt</sup> in FGF21KO.**

**(A)** COX/SDH histochemical activities and pS6 immunohistochemistry on consecutive frozen sections. Scale bar 50  $\mu$ m. Phosphorylated S6 protein is induced in the same fibers that are COX-SDH deficient (circled)

**(B)** ISR<sup>mt</sup> components and *Atf3-5* are expressed in MEFs as a consequence of mitochondrial translation inhibition (actinonin) independently of FGF21. Quantitative PCR analysis in WT and FGF21KO primary MEFs.

**(C)** Volcano plots of muscle metabolomes (Deletor, FKO n=6; WT, FKO n=8). Despite accumulation of acylcarnitine pool in Deletor-FKO (Del-FKO vs. FKO and Del-FKO vs. WT) the metabolic profile of Del-FKO mimics WT (Del-FKO vs. WT) rather than Deletor (DEL vs. Del-FKO). AMP = adenosine monophosphate, Asp = aspartate, GAA = guanidinoacetic acid, Glu = glutamate, IMP = inosine monophosphate, NAD = nicotinamide adenine dinucleotide, PE = phosphoethanolamine, Thr = threonine, TNO = trimethylamine N-oxide, 5-f-THF = formyl-THF, 5-m-THF=5-methyltetrahydrofolate. Acylcarnitines: carbon chain length followed by [C]=Carnitine.

Animal ages: 22-24 months old. Volcano plots: Statistical significance determined without correction for multiple comparisons,  $\alpha=0.05\%$  (horizontal line y-axis). Each metabolite analyzed individually, not assuming a consistent SD. Abbreviations: ATF=Activating transcription factor, Cth=Cystathionine gamma-lyase, COX=cytochrome-c-oxidase, Gdf15=Growth/differentiation factor 15, Mthfd2=methylenetetrahydrofolate-dehydrogenase-2 (mitochondrial), Phgdh=Phosphoglycerate dehydrogenase, Psat1=Phosphoserine aminotransferase, pS6=ribosomal S6-subunit phosphorylated form, SDH=succinate dehydrogenase



**Figure S5, related to Figure 5. Glucose uptake and pathology in CA2.**

**(A)** Digital autoradiography (ARG) of representative frozen sections, coronal slice coordinates visualized on the right (hematoxylin-eosin staining). Encircled area includes DHC; magnified in the lower panel, arrows point to increased PSL/mm<sup>2</sup> in DHC. N-numbers are 6-8 animals per group.

**(B)** Left: ARG of <sup>18</sup>F-FDG in brain of WT mice with intravenous recombinant FGF21 (rFGF21) injections or 24 h fasting. Arrows: DHC. Right: Serum FGF21 in animals of the experiment on the left.

**(C)** Long-range PCR analysis of mtDNA: Top: ~16 kb intact mtDNA and additional short smear-like products represent mtDNAs with multiple different deletions. Below: ~500 bp product of D-loop region, no mtDNA deletions.

**(D)** Astrocyte morphology in CA2, non-reactive. GFAP immunofluorescence, scale bar 100 μm.

**(E)** Microglial morphology in CA2, non-inflammatory. Immunofluorescence for IBA1. Arrows indicate the cells shown in greater magnification (white box). Scale bars 100 μm.

Animals were 22-24 months old, except in (B) 5 months. Bars represent the group average with SD. Box: 25th and 75th percentiles with median and range. Dots indicate data points from individual animals. Pairwise two-tailed t-test, statistical significance: \* p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001, \*\*\*\*p≤0.0001. Abbreviations: <sup>18</sup>F-FDG= Fluoro-deoxy-glucose, rFGF21=recombinant fibroblast growth factor 21, CA2=hippocampal CA-region 2, DHC=dorsal hippocampus, VHC=ventral hippocampus, GFAP=Glial fibrillary acidic protein, IBA1=ionized calcium binding adaptor molecule 1, DAPI=4',6-Diamidino-2-phenylindole (DNA dye), PSL=photostimulated luminescence, ΔmtDNA= mtDNA with deletions.

**Table S1. Related to Figure 1A. Average (FC), SD and n-number presented for each gene and comparison. Artificial units, WT average = 1. Highlighted in red: statistically significant difference ( $p < 0.05$ )**

MONTHS	6		9		12-15		16-18		21-22	
GENOTYPE	WT	DEL	WT	DEL	WT	DEL	WT	DEL	WT	DEL
<b>Gdf15</b>										
N	6	4	7	5	5	6	6	6	7	7
Mean	1.00	1.38	1.00	2.83	1.00	26.01	1.00	82.05	1.00	121.8
SD	0.59	0.58	0.47	0.73	0.37	30.2	0.55	31.74	0.63	92.16
<b>Fgf21</b>										
N	5	5	5	5	6	5	5	6	5	6
Mean	1.00	1.22	1.00	2.27	1.00	42.50	1.00	126.10	1.00	113.50
SD	1.13	0.92	1.44	3.91	0.62	28.48	0.72	108.4	0.66	99.98
<b>Mthfd2</b>										
N	7	5	5	6	6	5	5	6	5	6
Mean	1.00	0.83	1.00	1.60	1.00	9.02	1.00	11.86	1.00	19.27
SD	0.56	0.38	0.62	1.22	0.25	12.39	0.22	10.1	0.20	12.36
<b>Phgdh</b>										
N	7	5	5	5	6	5	5	6	5	6
Mean	1.00	1.93	1.00	1.33	1.00	0.63	1.00	1.04	1.00	4.20
SD	0.87	1.38	0.15	0.75	0.66	0.25	0.22	0.40	0.25	2.46
<b>Psat1</b>										
N	7	5	5	6	6	5	5	6	5	6
Mean	1.00	1.36	1.00	1.04	1.00	1.77	1.00	2.66	1.00	9.31
SD	0.47	0.65	0.20	0.40	0.32	0.78	0.16	0.95	0.08	6.09
<b>Hsp10</b>										
N	7	5	5	6	6	5	5	6	5	6
Mean	1.00	0.28	1.00	0.87	1.00	1.49	1.00	1.61	1.00	1.48
SD	1.04	0.10	0.18	0.30	0.23	1.14	0.20	0.93	0.18	0.84
<b>Hsp60</b>										
N	7	5	5	6	6	5	5	6	5	6
Mean	1.00	0.93	1.00	0.85	1.00	1.06	1.00	1.18	1.00	1.20
SD	0.14	0.18	0.09	0.31	0.04	0.19	0.16	0.18	0.08	0.28
<b>Hsp70</b>										
N	7	5	5	6	6	5	5	6	5	6
Mean	1.00	1.10	1.00	0.83	1.00	1.05	1.00	1.39	1.00	1.44
SD	0.30	0.19	0.17	0.39	0.08	0.21	0.14	0.26	0.17	0.38
<b>Atf3</b>										
N	6	6	5	5	6	6	5	6	5	7
Mean	1.07	0.73	1.10	0.91	1.00	1.20	1.00	1.09	1.00	2.51
SD	1.41	0.42	0.76	1.26	0.71	0.76	0.63	0.70	0.44	2.19
<b>Atf4</b>										
N	7	5	5	5	6	6	5	6	5	7
Mean	1.00	0.86	0.89	1.50	1.00	0.82	1.00	1.27	1.00	1.39
SD	0.59	0.16	0.19	0.98	0.35	0.35	0.24	0.41	0.24	0.71
<b>Atf5</b>										
N	7	5	5	5	6	6	5	6	5	7
Mean	0.93	0.88	1.00	1.25	1.00	1.64	1.00	2.68	1.00	3.74
SD	0.65	0.44	0.64	0.68	0.38	1.27	0.78	1.40	0.36	2.67

**Table S2. Related to Figure 1G. Differential gene expression analysis of representative ISR<sup>mt</sup>, UPR<sup>mt</sup> and ATF -genes in human mitochondrial myopathy. Standard error (SE). Significantly changed genes are highlighted with colours.**

GENE	Fold Change Patients vs. controls	SE low Value	SE upper Value	p value
MTHFD1	1.01	0.92	1.10	0.926
MTHFD1L	3.07	1.86	5.08	0.026
MTHFD2	1.69	1.33	2.15	0.030
MTHFD2L	1.42	1.16	1.73	0.080
GDF15	82.67	35.79	190.95	1.34 x 10 <sup>-7</sup>
FGF21	378.73	127.85	1121.89	4.58 x 10 <sup>-8</sup>
TRIB3	16.66	9.23	30.09	1.94 x 10 <sup>-6</sup>
ASNS	2.80	1.71	4.58	0.037
PSAT1	2.95	1.78	4.87	0.032
PHGDH	3.17	2.12	4.74	0.004
SHMT1	0.69	0.60	0.81	0.017
SHMT2	1.55	1.30	1.85	0.012
LONP1	1.00	0.85	1.17	0.992
CLPP	0.83	0.69	1.00	0.316
DDIT3	1.57	1.35	1.81	0.002
HSPE1	1.04	0.91	1.20	0.769
HSPD1	1.08	0.92	1.26	0.646
HSPA9	1.15	1.01	1.31	0.296
ATF3	0.66	0.41	1.07	0.388
ATF4	0.93	0.82	1.04	0.515
ATF5	5.22	3.35	8.15	2.04 x 10 <sup>-4</sup>
ATF6	1.24	1.11	1.38	0.053

**Table S3. Related to Figure 3G AND 3H. Average (FC), SD and n-number presented. Artificial units, WT and FKO average = 1. Parallel comparison between [WT vs. DEL] and [FKO vs. DEL-FKO] not possible due to instrument calibration between the experiments.**

**<sup>18</sup>F-FDG**

	WT			DEL		
	FC	SD	N	FC	SD	N
SKL. MUSCLE	1.00	0.10	6	1.44	0.23	5
HEART	1.00	0.29	6	1.71	0.57	5
WAT <sub>sc</sub>	1.00	0.17	6	1.65	0.57	5
WHOLE BRAIN	1.00	0.15	6	0.71	0.05	5
LIVER	1.00	0.09	6	0.69	0.07	5
SERUM	1.00	0.12	6	0.71	0.10	5
PANCREAS	1.00	0.50	6	0.42	0.05	5
SPLEEN	1.00	0.24	6	0.66	0.13	5
LUNGS	1.00	0.08	6	1.08	0.12	5
KIDNEYS	1.00	0.11	5	0.86	0.25	5
BAT	1.00	0.26	5	0.90	0.19	5
WAT <sub>ip</sub>	1.00	0.15	6	0.99	0.27	5

**<sup>18</sup>F-FDG**

	FKO			DEL-FKO		
	FC	SD	N	FC	SD	N
SKL. MUSCLE	1.00	0.43	6	1.23	0.48	6
HEART	1.00	0.27	6	0.97	0.32	6
WAT <sub>sc</sub>	1.00	0.86	6	0.54	0.13	6
WHOLE BRAIN	1.00	0.22	6	0.78	0.11	6
LIVER	1.00	0.24	6	0.79	0.10	6
SERUM	1.00	0.41	6	0.88	0.20	6
PANCREAS	1.00	0.25	6	0.84	0.17	6
SPLEEN	1.00	0.10	6	0.85	0.10	6
LUNGS	1.00	0.13	6	0.84	0.14	6
KIDNEYS	1.00	0.18	6	0.75	0.10	6
BAT	1.00	0.61	6	0.77	0.48	6
WAT <sub>ip</sub>	1.00	0.29	5	1.08	0.31	6

**<sup>18</sup>F-FTHA**

	WT			DEL		
	FC	SD	N	FC	SD	N
SKL. MUSCLE	1.00	0.27	7	0.99	0.16	6
HEART	1.00	0.21	7	0.87	0.11	6
WAT <sub>sc</sub>	1.00	0.28	7	1.03	0.29	6
WHOLE BRAIN	1.00	0.23	7	0.97	0.21	6
LIVER	1.00	0.16	7	1.02	0.13	6
SERUM	1.00	0.09	7	1.11	0.18	6
PANCREAS	1.00	0.27	7	1.05	0.19	6
SPLEEN	1.00	0.21	7	1.09	0.37	6
LUNGS	1.00	0.27	7	1.25	0.36	6
KIDNEYS	1.00	0.19	7	1.13	0.32	6
BAT	1.00	0.36	7	0.73	0.21	6
WAT <sub>ip</sub>	1.00	0.22	7	1.11	0.28	6

**<sup>18</sup>F-FTHA**

	FKO			DEL-FKO		
	FC	SD	N	FC	SD	N
SKL. MUSCLE	1.00	0.07	6	1.03	0.15	5
HEART	1.00	0.30	6	0.81	0.18	4
WAT <sub>sc</sub>	1.00	0.28	6	0.96	0.16	5
WHOLE BRAIN	1.00	0.21	6	1.06	0.43	5
LIVER	1.00	0.24	6	0.99	0.36	5
SERUM	1.00	0.23	6	1.08	0.47	5
PANCREAS	1.00	0.12	6	0.99	0.18	5
SPLEEN	1.00	0.44	6	1.28	0.52	5
LUNGS	1.00	0.36	6	0.91	0.37	5
KIDNEYS	1.00	0.39	6	1.05	0.54	5
BAT	1.00	0.18	6	1.01	0.13	5
WAT <sub>ip</sub>	1.00	0.39	6	0.73	0.19	5

**Table S4. Related to Figure 4E. Average (FC), SD and n-number presented with t-tests between genotypes. Artificial units, WT average = 1. In figure stars represented p-values of DEL vs. WT and DEL-FKO vs. WT.**

	WT			DEL			FKO			DEL-FKO			P-value (t-test)			
	FC	SD	N	FC	SD	N	FC	SD	N	FC	SD	N	DEL vs. WT	Del-FKO vs. WT	FKO vs. WT	DEL-FKO vs. FKO
GLUTAMATE	1.00	0.39	8	1.90	0.18	6	0.97	0.38	8	1.33	0.34	6	<b>0.0002</b>	<b>0.128</b>	0.872	0.096
GLYCINE	1.00	0.36	8	1.47	0.54	6	0.93	0.24	8	1.40	0.42	6	<b>0.072</b>	<b>0.081</b>	0.675	0.022
GLUTAMINE	1.00	0.34	8	1.35	0.36	6	0.72	0.21	8	1.11	0.33	6	<b>0.089</b>	<b>0.548</b>	0.066	0.018
ASPARAGINE	1.00	0.39	8	1.31	0.21	6	0.78	0.14	7	1.46	0.37	6	<b>0.102</b>	<b>0.045</b>	0.178	0.001
THREONINE	1.00	0.25	8	1.27	0.19	6	0.82	0.16	8	0.90	0.05	6	<b>0.047</b>	<b>0.365</b>	0.115	0.270
PROLINE	1.00	0.33	8	1.27	0.36	6	0.90	0.05	7	1.02	0.13	6	<b>0.163</b>	<b>0.886</b>	0.430	0.046
ASPARTATE	1.00	0.28	8	1.26	0.37	6	0.86	0.18	7	1.28	0.28	6	<b>0.169</b>	<b>0.096</b>	0.276	0.009
VALINE	1.00	0.26	8	1.19	0.22	6	0.87	0.15	8	1.02	0.10	6	<b>0.165</b>	<b>0.892</b>	0.223	0.059
SERINE	1.00	0.35	8	1.19	0.14	6	0.88	0.22	8	1.08	0.12	6	<b>0.244</b>	<b>0.600</b>	0.444	0.071
METHIONINE	1.00	0.27	8	1.17	0.25	6	0.92	0.14	8	1.02	0.11	6	<b>0.269</b>	<b>0.839</b>	0.471	0.157
LEUCINE	1.00	0.29	8	1.13	0.23	6	0.89	0.20	8	0.99	0.12	6	<b>0.368</b>	<b>0.922</b>	0.411	0.338
ISOLEUCINE	1.00	0.19	8	1.12	0.18	6	0.89	0.15	8	1.12	0.22	6	<b>0.249</b>	<b>0.300</b>	0.210	0.038
HISTIDINE	1.00	0.22	8	1.11	0.17	6	1.05	0.25	8	1.11	0.24	6	<b>0.343</b>	<b>0.375</b>	0.649	0.659
CYSTEINE	1.00	0.18	8	1.11	0.14	6	0.94	0.23	8	1.05	0.11	6	<b>0.264</b>	<b>0.575</b>	0.567	0.301
PHENYLALA.	1.00	0.23	8	1.09	0.18	6	0.90	0.16	8	0.95	0.06	6	<b>0.478</b>	<b>0.618</b>	0.331	0.484
TRYPTHOPHAN	1.00	0.16	8	1.07	0.25	6	0.90	0.24	8	1.03	0.14	6	<b>0.564</b>	<b>0.681</b>	0.365	0.257
ARGININE	1.00	0.21	7	1.03	0.26	6	0.78	0.17	8	1.21	0.38	6	<b>0.836</b>	<b>0.227</b>	0.041	0.013
TYROSINE	1.00	0.31	8	1.03	0.34	6	0.96	0.20	8	1.10	0.21	6	<b>0.881</b>	<b>0.522</b>	0.776	0.250
ALANINE	1.00	0.29	8	1.01	0.14	6	0.80	0.18	8	1.09	0.17	6	<b>0.934</b>	<b>0.503</b>	0.115	0.009

**Table S5. Related to Figure 4F. Average (FC), SD and n-number presented with t-tests between genotypes. Artificial units, WT average = 1. In figure stars represented p-values of DEL vs. WT and DEL-FKO vs. WT.**

	WT			DEL			FKO			DEL-FKO			P-value (t-test)			
	FC	SD	N	FC	SD	N	FC	SD	N	FC	SD	N	DEL vs. WT	Del-FKO vs. WT	FKO vs. WT	DEL-FKO vs. FKO
Carnitine	1.00	0.33	8	1.34	0.19	6	0.93	0.42	8	1.13	0.20	6	<b>0.0432</b>	<b>0.3998</b>	0.7008	0.2907
Acetyl-carnitine	1.00	0.22	8	1.32	0.28	6	0.81	0.34	8	0.91	0.18	6	<b>0.0309</b>	<b>0.4077</b>	0.2111	0.5495
Propionyl-carnitine	1.00	0.30	8	1.12	0.33	6	1.07	0.45	8	1.02	0.20	6	<b>0.4830</b>	<b>0.9073</b>	0.7048	0.7788
Isovaleryl-carnitine	1.00	0.38	8	1.18	0.62	6	1.45	0.52	8	1.21	0.44	6	<b>0.5211</b>	<b>0.3571</b>	0.0678	0.3766
Hexanoyl-carnitine	1.00	0.37	8	1.45	0.40	6	0.81	0.37	8	1.15	0.38	6	<b>0.0530</b>	<b>0.4701</b>	0.3216	0.1152
Octanoyl-carnitine	1.00	0.34	8	1.44	0.33	6	0.91	0.30	8	1.27	0.24	6	<b>0.0342</b>	<b>0.1247</b>	0.5983	0.0328
Decanoyl-carnitine	1.00	0.51	8	1.29	0.24	6	0.81	0.31	8	1.28	0.32	6	<b>0.2212</b>	<b>0.2668</b>	0.3774	0.0171
Dodecanoyl-carnitine	1.00	0.63	8	1.42	0.56	6	0.74	0.41	8	2.00	0.98	6	<b>0.2185</b>	<b>0.0390</b>	0.3533	0.0066
Tetradecanoyl-carnitine	1.00	0.72	8	1.45	0.72	6	0.67	0.46	8	2.20	1.26	6	<b>0.2712</b>	<b>0.0431</b>	0.2982	0.0079
Palmitoyl-carnitine	1.00	0.79	8	1.93	1.44	6	0.50	0.18	7	3.02	1.84	6	<b>0.1469</b>	<b>0.0160</b>	0.1287	0.0040
Isobutyryl-L-carnitine	1.00	0.37	8	1.28	0.33	6	0.76	0.32	8	0.99	0.25	6	<b>0.1712</b>	<b>0.9405</b>	0.1836	0.1737
Stearoyl-carnitine	1.00	0.62	8	1.36	0.80	6	0.69	0.44	8	2.07	1.28	6	<b>0.3568</b>	<b>0.0588</b>	0.2772	0.0143
Arachidyl-carnitine	1.00	0.66	8	1.36	1.18	6	0.64	0.40	8	2.38	2.12	6	<b>0.4730</b>	<b>0.1052</b>	0.2091	0.0405

**Table S6. Related to STAR METHODS section. Human and mouse primer sequences.**

Mouse genes		5'-3'	Human genes		5'-3'
<b>Atf1</b>	Fwd	CGACAGAGACTGCATCTCAGCC	<b>ATF3</b>	Fwd	ATTTTGCTAACCTGACGCC
	Rev	CCGTGAGCTTTCTGTGAGGAG		Rev	CCGTCTTCTCCTTCTTGTTCG
<b>Atf2</b>	Fwd	TGTCATTGTGGCTGATCAGACTC	<b>ATF4</b>	Fwd	GTCAGTCCCTCCAACAACAGC
	Rev	GTGTTGCAAGAGGGGACAAATC		Rev	TCCAACGTGGTCAGAAGGTCATC
<b>Atf3</b>	Fwd	CCAGAATAAACACCTCTGCCATCG	<b>ATF5</b>	Fwd	CAGCCATGGAGTCTCCACTTTC
	Rev	CTTCAGCTCAGATTCACTCTC		Rev	GGCACTGATGGCAACAGGAGAG
<b>Atf4</b>	Fwd	CTCATGGGTTCTCCAGCGACAAG	<b>FGF21</b>	Fwd	ACCTGGAGATCAGGGAGGAT
	Rev	GTC AAGAGCTCATCTGGCATGG		Rev	GCACAGGAACCTGGATGTCT
<b>Atf5</b>	Fwd	GAGGGAGGTCTCGTGTACGTCTG	<b>GDF15</b>	Fwd	AGAGCTGGGAAGATTGGAACAC
	Rev	GCTTCTCAGTTGCACTGAAGGGG		Rev	CGGAACAGAGCCCGGTGAAG
<b>Atf6</b>	Fwd	CTTGGGAGTCAGACCTATGGAG	<b>MTHFD2</b>	Fwd	TGAAGAGCGAGAAGTGCTGA
	Rev	TCAGGAACGTGCTGAGTTGAAG		Rev	GAATGCTCCCTGGTGAGGTA
<b>Atf7</b>	Fwd	TGATCCACTTATCCAACCTTCC	<b>CTH</b>	Fwd	GATTGAAAGCCTTGCTGAG
	Rev	TGTCATTAGCAAGATGCATGACG		Rev	ACTTCCACTTGGAGGGTGTG
<b>Phgdh</b>	Fwd	GACCCCATCATCTCTCTGA	<b><math>\beta</math>-Actin</b>	Fwd	CCTGGCACCCAGACAAT
	Rev	GCACACCTTTCTTGCCTGA		Rev	GGCCGGACTCGTCATAC
<b>Cth</b>	Fwd	TGCTAAGGCCTTCTCAAAA			
	Rev	GTCCTTCTCAGGCACAGAGG			
<b>Clpp</b>	Fwd	CTGCCCAATTCCAGAATCAT			
	Rev	TGTAGGCTCTGCTTGGTGTG			
<b>mtHsp10</b>	Fwd	CTTTCCTTCCCTCCTGACC			
	Rev	CAAAGGTGGCATTATGCTT			
<b>mtHsp60</b>	Fwd	TCCCTGCTCTTGAAATTGCT			
	Rev	GCTTCTCCGTCAACGTCTTC			
<b>mtHsp70</b>	Fwd	GCTGCTGATGGACAACTCA			
	Rev	GGGGGAATTCCAATCAAAGT			
<b><math>\beta</math>-Actin</b>	Fwd	ATGCTCCCCGGGCTGTAT			
	Rev	CATAGGAGTCTTCTGACCCATTC			
<b>Trib3</b>	Fwd	TCGCTTTGTCTTCACTCACTGTGAG			
	Rev	CATCAGCCGCTTGGCAGAGTAG			
<b>Mthfd2</b>	Fwd	CATGGGGCATATGGGAGATAAT			
	Rev	CCGGGCCGTTTCGTGAGC			
<b>Asns</b>	Fwd	CTGCTTTGGCTTTCACCGCTTG			
	Rev	TGCTGTAGCGCCTTGTGGTTG			
<b>Gdf15</b>	Fwd	CAACCAGAGCCGAGAGGAC			
	Rev	TGCACGCGGTAGGCTTC			
<b>XBP1 splicing</b>	Fwd	GAACCAGGAGTTAAGAACACG			
	Rev	AGGCAACAGTGTGAGATCC			

Mouse mtDNA analysis		5'-3'
<b>mtDNA Triplex (ND4)</b>	Fwd	CCATTCTCCTCTATCCCTCAAC
	Rev	CACAATCTGATGTTTTGGTTAACTATATTT
	Probe	6FAM-CCGACATCATTACCGGGTTTTCTCTTG
<b>mtDNA Triplex (D-loop)</b>	Fwd	CCCACACGTTCCCTTAAATAA
	Rev	CGTGAGTGGTTAATAGGGTGATAGAC
	Probe	ROX-ACATCACGATGGATCAC
<b>mtDNA deletions (long PCR)</b>	Fwd	GAGGTGATGTTTTTGGTAAACAGGCGGGT
	Rev	GGTTCGTTTGTCAACGAT TAAAGTCTACGTG
<b>mtDNA intact (short PCR)</b>	Fwd	ACCCCGCCTGTTTACCAAAAACATCACCTC
	Rev	ACGTACCACTTAATCGTTGAACAAACGAACC