Skeletal muscle mitochondrial uncoupling drives endocrine cross-talk through the induction of FGF21 as a myokine

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Keipert S, Ost M, Johann K, Imber F, Jastroch M, van Schothorst EM, Keijer J, Klaus S. Skeletal muscle mitochondrial uncoupling drives endocrine cross-talk through the induction of FGF21 as a myokine. Am J Physiol Endocrinol Metab 306: E469–E482, 2014. First published December 17, 2013; doi:10.1152/ajpendo.00330.2013.—UCP1-Tg mice with ectopic expression of uncoupling protein 1 (UCP1) in skeletal muscle (SM) are a model of improved substrate metabolism and increased longevity. Analysis of myokine expression showed an induction of fibroblast growth factor 21 (FGF21) in SM, resulting in approximately fivefold elevated circulating FGF21 in UCP1-Tg mice. Despite a reduced muscle mass, UCP1-Tg mice showed no evidence for a myopathy or muscle autophagy deficiency but an activation of integrated stress response (ISR; eIF2α/ATF4) in SM. Targeting mitochondrial function in vitro by treating C2C12 myoblasts with the uncoupler FCCP resulted in a dose-dependent activation of ISR, which was associated with increased expression of FGF21, which was also observed by treatment with respiratory chain inhibitors antimycin A and myxothiazol. The cofactor requirement for FGF21 action, β-klotho, was expressed in white adipose tissue (WAT) of UCP1-Tg mice, which showed an increased browning of WAT similar to what occurred in altered adipocyte morphology, increased brown adipocyte markers (UCP1, CIDEA), lipolysis (HSL phosphorylation), and respiratory capacity. Importantly, treatment of primary white adipocytes with serum of transgenic mice resulted in increased UCP1 expression. Additionally, UCP1-Tg mice showed reduced body length through the suppressed IGF-I-GH axis and decreased bone mass. We conclude that the induction of FGF21 as a myokine is coupled to disturbance of mitochondrial function and ISR activation in SM. FGF21 released from SM has endocrine effects leading to increased browning of WAT and can explain the healthy metabolic phenotype of UCP1-Tg mice. These results confirm muscle as an important endocrine regulator of whole body metabolism.

ECTOPIC EXPRESSION IN SKELETAL MUSCLE (SM) OF uncoupling protein 1 (UCP1), the mitochondrial uncoupling protein of brown adipose tissue (BAT), leads to increased energy expenditure, delayed diet-induced obesity development, reduced hepatic steatosis, improved glucose homeostasis, and increased longevity in these muscle-specific UCP1-transgenic (Tg) mice (22, 34, 38, 50). The exact physiological mechanisms underlying this metabolic improvement have not yet been resolved. Respiratory uncoupling affected the function of not only muscle itself but also white adipose tissue (WAT), which showed an increased metabolic activity and increased insulin-stimulated glucose uptake (32, 50). This suggests cross-talk between muscle and WAT that is possibly mediated by myokines. Muscle is considered a secretory organ, secreting several hundred products. It is suggested that the beneficial health effects of exercise training could be mediated in part by secretion of such myokines that could counteract harmful effects of proinflammatory cytokines released, e.g., by WAT (52). This is supported by the recent identification of the novel myokine irisin, which is released during exercise and leads to the induction of UCP1 expression and a BAT-like development in WAT linked to improvements in obesity and glucose homeostasis in mice (6). As in exercise, UCP1 induced SM respiratory uncoupling leads to an activation of AMP-activated protein kinase (AMPK) (50) mediating increased fatty acid oxidation in muscle (33).

Fibroblast growth factor 21 (FGF21) is a unique member of the FGF superfamily subgroup that also includes FGF19/15 and FGF23. Rather than being a growth factor, it is a pleotropic hormone-like circulating protein that functions as a major metabolic regulator of glucose and lipid metabolism (7, 27, 35, 49). Circulating FGF21 is thought to be secreted primarily by the liver (3, 51). Hepatic FGF21 expression and secretion are induced largely by fasting and ketogenic diets through activation of PPARα and play an important role in the adaptation to starvation (15, 28, 41). The biological activity of FGFs is dependent on their binding to FGF receptors (FGFR) and the coreceptor β-Klotho (KLB). KLB is crucial for FGF21 specificity (27) and its metabolic action (2, 14). FGF21 expression in muscle is induced by hyperinsulinemia in humans (26), and it has been suggested to be a novel insulin-stimulated myokine (52). Interestingly, FGF21 overexpression in mice leads to a phenotype that very much resembles that of UCP1-Tg mice with ectopic UCP1 in SM, i.e., a reduced body weight and size despite normal or even increased food intake, improved glucose homeostasis (29, 36), and increased energy expenditure and longevity (63). Systemic administration of FGF21 was further shown to reduce diet-induced as well as genetic obesity (8) and is being considered as a potential therapeutic in obesity, diabetes, and related disorders.

Here, we aimed to explore whether the healthy metabolic phenotype of Tg mice expressing UCP1 in SM is linked to an induction and release of myokines, focusing on FGF21. We show that expression and secretion of FGF21 are highly induced in the SM of UCP1-Tg mice, leading to more than fivefold increased circulating levels of FGF21. This is accom-
compared by an increased browning and substantially increased respiratory capacity of WAT depots.

MATERIALS AND METHODS

Animals. UCPI1-transgenic (UCPI1-Tg) animals were generated as described previously (38). Experiments were performed in adult male hemizygous UCPI1-Tg and their wild-type (WT) littermates. Mice were housed in groups with ad libitum access to food and water at 23°C and a 12:12-h dark-light cycle. From 12 wk of age, mice were fed a low-fat diet (LFD) or a high-fat diet (HFD) (32) for 8 or 30 wk. Unless otherwise stated, all data are from adult male mice of ~20 wk of age. Animal maintenance and experiments were approved by the ethics committee of the Ministry of Agriculture and Environment (State of Brandenburg, Germany; permission no. GZ 23-2347-8-2009).

Body composition and bone mineral density. Body composition was determined by quantitative magnetic resonance (Bruker’s Minispec MQ10, Houston, TX), and bone mineral density was determined by dual-energy X-ray absorptiometry (Lunar Piximus).

Gene expression analysis. RNA isolation and quantitative real-time PCR (qPCR) were performed as described before (33). Quadriceps and gastrocnemius combined were used for analysis of SM expression. Multiplex gene expression was calculated as $\Delta C_T$, using β-actin for normalization. Tissue-specific gene expression was calculated as $\Delta \Delta C_T$, using β-actin or β2 microglobulin (B2M) for normalization and relative to the WT group, which was normalized to a value of 1. The oligonucleotide primer (probe) sequences are listed in Table 1.

Growth factor analysis. FGF21 (mouse/rat FGF21 Quantikine ELISA Kit; R & D Systems), GH (rat/mouse growth hormone ELISA; Millipore), and IGF-I (mouse/rat IGF-I Quantikine ELISA Kit; R & D Systems) values were determined by ELISA, following the manufacturers’ instructions.

Citrate synthase and cytochrome c oxidase activity assay. Citrate synthase (CS) activity was determined spectrophotometrically, as described previously (55), by monitoring the formation of DTNB at 412 nm. Briefly, muscle tissue was homogenized in 50 mM Tris, 1 mM EDTA (pH 8.2), and 0.1% Triton X-100 and centrifuged at 13,000 g for 10 min or with 4 μM antimycin A (Sigma) and 4 μM myxothiazol (Sigma) for 8 h (n = 3–5 biological replicates).

Murine WAT cell culture, differentiation, and treatment. The subcutaneous WAT pads of five WT mice (7 wk old) were dissected, minced, and digested (50 min at 37°C at 100 rpm in DMEM-F12 plus glutamax containing 0.15% collagenase type IV (Life Technologies) and 2% BSA). The digested tissue was then filtered through a 40-μm cell strainer and centrifuged at 400 g for 10 min to pellet the stromal vascular (SV) fraction. The SV cells were then resuspended in erythrocyte-lysis buffer (154 mM NaCl, 10 mM KHCO3, and 0.1 mM EDTA), incubated for 20 min at room temperature, and centrifuged at 400 g for 10 min. SV cells were resuspended in growth medium (DMEM-F12 plus glutamax, 1% penicillin-streptomycin, and 10% BSA) and again filtered through a 40-μm cell strainer and plated on a T75 flask. For differentiation, SV cells were plated in 12-well plates, allowing them to grow to 90–100% confluence. At confluence, differentiation was started using a white fat differentiation cocktail as described previously (19) containing growth medium with 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 5 μg/ml insulin, and 1 μM rosiglitazone. On day 3 of differentiation, cells were exposed to DMEM-F12 plus glutamax, 1% penicillin-streptomycin, and 20% WT or UCPI1-Tg serum both with and without 2 μg of mouse FGF21 antibody (R & D Systems) for another 5 days (with 1 culture medium change after 3 days). On day 7 of differentiation, cells were harvested, and RNA was isolated (Qiagen-RNeasy Lipid Tissue Kit) for gene expression analyses.

Statistical analysis. Statistical analyses were performed using Graph Prism (5.0) (GraphPad, San Diego, CA). Data are reported as means ± SE. Two-tailed Student’s t-test was used to determine differences between the genotype. One-way ANOVA was used to area was performed using Adiposoft (1.0; Center for Applied Medical Research of the University of Navarra, Spain) (5). Major outcomes are presented as mean ± SE. Two-tailed Student’s t-test was used to determine differences between the genotypes. One-way ANOVA was used to...
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<th>Gene</th>
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| β-Actin                                   | ACTB    | **Forward:** TGT TAC CAA CTG GGA CGA CA  
|                                            |         | **Reverse:** GGG GTC TGG AAG CTC TGA AA  
|                                            |         | **Probe:** ACC GTC AAA AGA TGA CGG AG |
| Acetyl-coenzyme A carboxylase-α           | ACACA   | **Forward:** TTT CAT CGT GCC TCC AG  
|                                            |         | **Reverse:** TGC TAA TCA CTG CGA TA  
|                                            |         | **Probe:** CCA TTT TGG GCG ATA AGA ACC TCC TCA |
| Patatin-like phospholipase domain containing 2 | PNPLA2  | **Forward:** TTA CTA GGA GCA GCC ATG GA  
|                                            |         | **Reverse:** TGA TTA TCC TGC CCT GTG TA  
|                                            |         | **Probe:** CTG CAG CAG CTG TGG CCT CA |
| Fatty acid synthase                       | FASN    | **Forward:** TTT GCA GCA GCC AGC AG  
|                                            |         | **Reverse:** TCC CCT CTC ATG GCC AGC GA  
|                                            |         | **Probe:** CTG CAG CAG CTG TGG CCT CA |
| Hormone-sensitive lipase                  | LIPE    | **Forward:** TGC TGG TCC AAG TCG AGA GC  
|                                            |         | **Reverse:** TCG TCT TGT CCC TGA ATA AG  
|                                            |         | **Probe:** CTG TGA GCC GAA GGG ACA CA |
| Phosphoenolpyruvate carboxykinase 1       | PEPCK1  | **Forward:** TTT GCA GCA GCC AGC AG  
|                                            |         | **Reverse:** GCC GGT TCC CAC TTG GAA TA  
|                                            |         | **Probe:** ACA CGA TGC TGG CCT CCC TG |
| Peroxisome proliferator-activated receptor-α | PPARA   | **Forward:** ATT CGG CTG AAG CTG GTG TA  
|                                            |         | **Reverse:** AAG CGA ATT GCA TTG TGT GA  
|                                            |         | **Probe:** CGA CAA TGT TGA TCC GAG CGA CA |
| Peroxisome proliferator-activated receptor-γ | PPARG   | **Forward:** GCC GGT TCC CAC TGC TGA GAA TA  
|                                            |         | **Reverse:** ACA CGA TGC TGG CCT CCC TG  
| Peroxisome proliferator-activated receptor-γ coactivator-1α | PPARGC1A | **Forward:** CCT GAG ACA CCA CA CAC AC  
|                                            |         | **Reverse:** GCC AAG CGT GAA AAG AGA C  
|                                            |         | **Probe:** TAC GAC CAG AGT CAT ACA G |
| Uncoupling protein 1                      | UCP1    | **Forward:** ATT CGG CTG AAG CTG GTG TA  
|                                            |         | **Reverse:** AAG CGA ATT GCA TTG TGT GA  
|                                            |         | **Probe:** CGA CAA TGT TGA TCC GAG CGA CA |
| β-2-microglobulin                         | B2M     | **Forward:** GCC TCT TCA TCA CCA AGC AG  
|                                            |         | **Reverse:** AAA GAG ACC CTG COT GAA CA  
|                                            |         | **Probe:** GCC TCT TCA TCA CCA AGC AG |
| Cell death-inducing DNA fragmentation factor, α-subunit-like effector A | CIDEA   | **Forward:** TGC CAA AAA TAT CCC TGG TT  
|                                            |         | **Reverse:** GCC GGT TCC CAC TGC TGA GAA TA  
|                                            |         | **Probe:** ACA CGA TGC TGG CCT CCC TG |
| DNA-damage inducible transcript 3          | DDIT3   | **Forward:** CCA CCC CTG CAT TCC ACT TTC C  
|                                            |         | **Reverse:** TCC CCT TTG ACT GCC TCC TCA CTG GTG  
| Fibroblast growth factor 21               | FGF21   | **Forward:** AGA ATG GCC GGC AGA GAG AAG  
|                                            |         | **Reverse:** TCC CGG AAA AGG CAT CCT |
| Fibroblast growth factor receptor 1        | FGFR1   | **Forward:** TGT TTG AGG GGA TGT ACG ACA GAC A  
|                                            |         | **Reverse:** CTCCCCAGAAAGACTCCCCAA |
| Fibroblast growth factor receptor 2        | FGFR2   | **Forward:** TGC TGA TCC AAG CTG TGA AG  
|                                            |         | **Reverse:** GGG GAC CAC ACT TCC CAT AA  
| Fibronectin type III domain-containing 5   | FNDC5   | **Forward:** ATG AAG GAG ATG GGG AGG AA  
|                                            |         | **Reverse:** GCC GCA GAA GAG AGC CAT AAC A  
| Protein phosphatase 1 regulatory (inhibitor) subunit 15A | PPP1R15a | **Forward:** GCC GTG TTA AGG AAT CAT CTG  
|                                            |         | **Reverse:** AGA GTG TGG ACT GGG CAG C  
| Interleukin-15                            | IL-15   | **Forward:** GCC GCC GGC ATC AGG CAA CA  
|                                            |         | **Reverse:** GCC GTG TTA AGG AAT CAT CTG  
| Interleukin-6                             | IL-6    | **Forward:** CTC AGA GAG GTG AGG AAA GAA TCC  
|                                            |         | **Reverse:** GAC CAT GAA GAG GCA GTG CTG  
| β-Klotho                                  | KLB     | **Forward:** TTG TCT CTT GTC CCT CCC CAC  
|                                            |         | **Reverse:** ACT GAA GGA TCC CAC TGT GCA  
| Leucine-rich α2-glycoprotein 1            | LG1     | **Forward:** GCA TGC TCC GCT GTG ACT G  
|                                            |         | **Reverse:** TTG GAG AAT TCC ACC GAC AG |
| Tissue inhibitor of metalloproteinase 4    | TIMP4   | **Forward:** GCC CCT GCC GCC AGC ACT GAA AC  
|                                            |         | **Reverse:** GGA GAC CAC ACT TCC CCT |
| Uncoupling protein 1 (native)              | native UCP1 | **Forward:** CTT GCA GCA GCA TCC GCT TTA  
|                                            |         | **Reverse:** TGAGCA GAG CAG GCA GCA GCA  
| Vascular endothelial growth factor-β      | VEGFB   | **Forward:** TAT CCA GCA GAG TGG ATG TGG  
|                                            |         | **Reverse:** GCC AGA AGA TGC TGA CCT GAC |
evaluate differences between groups with appropriate post hoc tests. Statistical significance was assumed at $P < 0.05$.

RESULTS

FGF21 expression and increased secretion from SM in UCP1-Tg mice. Real-time RT-PCR gene expression analysis of relevant myokines (Fig. 1A) showed a massive increase of FGF21 in SM of UCP1-Tg mice compared with WT mice. Of the other myokines investigated, only vascular endothelial growth factor-B and interleukin 6 (IL-6) showed significant but much lower increased expression in UCP1-Tg muscle. FGF21 is considered to be preferentially expressed in liver (51), but it is also found in other metabolic tissues such as WAT and BAT (47). We examined FGF21 gene expression in several metabolic tissues in WT mice and observed that it was highest in liver and much lower in inguinal subcutaneous WAT (sWAT) and SM (Fig. 1B). We also confirmed that the induction of FGF21 in UCP1-Tg mice was specific to SM, whereas FGF21 mRNA was ~50-fold higher than in liver (Fig. 1B).

Fig. 1. Fibroblast growth factor 21 (FGF21) expression and increased secretion from skeletal muscle (SM) in uncoupling protein 1-transgenic (UCP1-Tg) mice. A and B: quantitative PCR (qPCR) analysis of myokines in SM (A) and FGF21 in SM, liver, and subcutaneous WAT (sWAT) of wild-type (WT) and UCP1-Tg mice (B). C: plasma FGF21 concentration of WT and UCP1-Tg mice fed a low-fat diet (LFD) or high-fat diet (HFD) since 12 wk of age in mice aged 20 (left) and 42 wk (right). D: ex vivo secretion of FGF21 (pg/ml) from extensor digitorum longus (EDL) or soleus muscle of WT and UCP1-Tg mice after 2-h incubation normalized to muscle wet weight (mg). *: gene expression and representative immunoblots of EDL or soleus muscle from WT and UCP1-Tg mice after 2-h incubation normalized to muscle wet weight (mg). E: expression and representative immunoblots of EDL or soleus muscle from WT and UCP1-Tg mice (α-tubulin was used as a control). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$, significant differences between the genotypes; $n = 6–8$ group. ND, not detectable. Data are means ± SE. FNDC, fibronectin type III domain-containing 5; LRG1, leucine-rich α2-glycoprotein 1; TIMP4, tissue inhibitor of metalloproteinase 4.

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Analysis of plasma FGF21 levels showed around fivefold increased plasma levels in UCP1-Tg mice compared with WT mice and was in the range of 5–7 ng/ml (Fig. 1C). This is slightly higher than previously reported fasting levels in mice but lower than plasma levels of FGF21 transgenic mice, which were ~20 ng/ml (29). We further investigated the effect of age and diet on FGF21 plasma levels of mice fed HFD or LFD until they reached the age of 20 (Fig. 1C, left) or 42 wk (Fig. 1C, right). In a previous study (33), we could show that WT mice developed obesity accompanied by increased liver triglycerides and elevated plasma insulin levels already at 20 wk. UCP1-Tg mice were not affected by HFD at 20 wk but became obese in older age. Interestingly, young UCP1-Tg mice showed highly increased FGF21 plasma levels compared with WT on both diets, being even higher on the HFD (Fig. 1C). In older age (42 wk), FGF21 plasma levels decreased slightly in UCP1-Tg mice. In young WT mice there was no effect of diet on FGF21 plasma levels, but it was elevated (albeit not significantly) by HFD feeding in older mice. The increased FGF21 levels on HFD agree with increased circulating FGF21 in obesity in different mouse models and humans (27). This is linked to a FGF21 resistance, evidenced by a large attenuation of FGF21 effects on liver and adipocytes (17) due possibly to an inflammation-induced repression of KLB expression, as shown in adipocytes (13).

**Fig. 2.** Reduced muscle mass without muscle dysfunction or features of myopathy in UCP1-Tg mice. A: representative hematoxylin and eosin staining of tibialis muscle from a WT and a UCP1-Tg mouse showing a general decrease in myofiber size but no different features of muscle degeneration, including central nuclei and vacuolated fibers. Scale bars, 50 μm. B: representative electron micrographs of WT and UCP1-Tg gastrocnemius muscles. C: quantification of cross-sectional area of myofibers; data from 3 mice in each group. D: locomotor activity [normal cage activity (left) and voluntary running wheel activity (right)] of WT and UCP1-Tg mice. E: citrate synthase (CS) activity in SM. ***P < 0.0001, significant differences between the genotypes; n = 5–8/group. Data are means ± SE.

MTI-CHONDRIAL UNCOUPLING INDUCES MUSCLE FGF21

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Moreover, secretion of FGF21 from SM was confirmed by 2-h ex vivo incubation of isolated SM (Fig. 1D). FGF21 concentrations were below the detection limit in medium from WT muscles but were very substantial in both soleus and EDL muscles from UCP1-Tg mice. FGF21 secretion was around sixfold higher in EDL, a fast-twitch, glycolytic type II muscle, than in soleus, a slow-twitch, oxidative, type I muscle. We detect the same pattern in FGF21 gene and protein expression, with higher expression in EDL compared with soleus muscle (Fig. 1E). This agrees with protection from the uncoupling effects of UCP1 of slow-twitch muscles because of their high continuous ATP synthesis (9) and suggests that the induction of FGF21 expression in SM is tightly linked to the actual mitochondrial uncoupling induced by UCP1.

**Reduced muscle mass without muscle dysfunction or features of myopathy in UCP1-Tg mice.** Until now, a possible contribution of muscle FGF21 secretion to circulating FGF21 was shown only in models of mitochondrial dysfunction and myopathy (37, 59). Therefore, we investigated in more detail UCP1-Tg muscle physiology and morphology. Histology showed reduced fiber size but no apparent degenerative changes in SM morphology, such as central nuclei (Fig. 2, A and C), misalignment of Z-line, or abnormal mitochondria (Fig. 2B). This is in line with previous findings, where we could show that there is no evidence for alterations in mitochondrial function and density despite a decreased muscle mass in UCP1-Tg mice compared with WT littermates (33). Physiological muscle function was also not impaired, as evident from recordings of normal cage activity (using infrared sensors) as well as voluntary running wheel activity. Both showed no differences between UCP1-Tg and WT mice (Fig. 2D). Furthermore, we also measured SM CS activity as a marker for mitochondrial density and could detect no differences between UCP1-Tg and WT mice (Fig. 2E).

**No basal autophagy defect in muscle of UCP1-Tg mice.** Autophagy is required for maintenance of normal muscle function (53), and suppression of SM autophagic flux was shown to lead to severe muscle dystrophy and mitochondrial dysfunction but also to an induction of FGF21 expression and release from muscle (37). Here, we assessed basal autophagy as nonstimulated autophagic flux by measuring protein levels of LC3-I (microtubule-associated protein 1 light chain 3-MAP1LC3A or LC3) and LC3-II (which is the phosphatidylethanolamine-conjugated and by this activated form of LC3-I) and the LC3-II/LC3-I ratio as marker of LC3-I to LC3-II conversion as well as the protein content of sequestosome 1 (SQSTM1 or p62) (31, 45, 46). p62 is one of the best characterized substrates of selective autophagy, which is incorporated via direct interaction with LC3 into the autophagosome and by this degraded during the autophagic process (30, 61). It is important to highlight that impairment of autophagy is accompanied by an accumulation of p62, as also observed in muscle-specific ATG7-knockout mice (37, 39). Therefore, we further analyzed the protein expression levels of BECLIN-1 and ATG7 as additional key proteins that are involved in autophagosome formation (25, 43). UCP1-Tg muscle showed elevated LC3-II protein levels, but without increased LC3-II/ LC3-I ratio and a trend of reduced p62 protein content (P = 0.06) compared with WT littermates (Fig. 3, A and B). Moreover, total LC3 (LC3-I + LC3-II), BECLIN-1, and ATG7 protein expression were increased in muscle of UCP1-Tg mice (Fig. 3, A and C). This is indicative for an induction of autophagic machinery, but without increased basal autophagy flux. However, these results clearly show that, in contrast to

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![Graph](image.png)

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**Fig. 3.** No basal autophagy defect in muscle of UCP1-Tg mice. A: representative immunoblots of key proteins involved in autophagy from SM of WT and UCP1-Tg mice. B: quantification of LC3-II, LC3-II/LC3-I ratio, and p62 protein collectively as markers of basal autophagy flux. C: quantification of BECLIN-1, autophagy-related protein 7 (ATG7), and total LC3 (LC3-I + LC3-II) proteins (α-tubulin was used as a control). **p < 0.01 and ***p < 0.0001, significant differences between the genotypes; n = 6/group. Data are means ± SE.
Altered mitochondrial respiration induces FGF21 in muscle cells. To examine the cellular mechanisms leading to FGF21 induction in more detail, we treated differentiated C2C12 cells with different compounds targeting mitochondrial function. This included the complex 3 inhibitors antimycin A and myxothiazol as well as the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP).

It is known that antimycin A, an inhibitor acting at the quinone reducing center (Qi), stimulates superoxide formation by inhibiting semi-quinone oxidation, whereas myxothiazol, an inhibitor of the Qo site, inhibits superoxide production by preventing semi-quinone formation (10, 58). Indeed, both mitochondrial stressors induced FGF21 gene expression in C2C12 cells (Fig. 5A), suggesting that FGF21 expression is activated in response to mitochondrial stress independent of reactive oxygen species (ROS) production. To determine whether uncoupling of the respiratory chain itself can lead to an induction of FGF21 gene expression in vitro, we furthermore treated differentiated C2C12 cells with the chemical uncoupler FCCP. C2C12 cells treated with different concentrations of FCCP for 24 h showed a dose-dependent increase in FGF21 gene expression (Fig. 5B). The induction of FGF21 was also confirmed on protein levels (Fig. 5C). As in skeletal muscle of UCP1-Tg mice, in C2C12 cells the increased FGF21 expression was associated with an activation of the eIF2α/ATF4 cascade.

Kim et al. (37), our mouse model has no signs of autophagy deficiency.

Activation of integrated stress response in muscle of UCP1-Tg mice. Although overall SM function is preserved in UCP1-Tg mice, we have previously found evidence for an increased cellular stress (33). Recent studies (37) have implicated the eIF2α/ATF4 cascade, which is part of the integrated stress response (ISR), in the induction of FGF21 gene expression in muscle. Real-time RT-PCR analysis showed highly increased expression of genes related to the ISR pathway in SM of UCP1-Tg mice (Fig. 4A). This included not only ATF4 but also ATF5 and ATF6 together with an increased expression of DNA damage-inducible transcript 3 (DDIT3 or CCAAT/enhancer-binding protein homologous protein) and protein phospatase 1 regulatory (inhibitor) subunit 15A (PPP1R15A or growth arrest and DNA damage-inducible protein 34), both of which are stress response genes known to be induced by ATF4 (42). Phosphorylation of eIF2α was highly increased in UCP1-Tg mice compared with WT mice (Fig. 4B), leading to the subsequent induction of ATF4 expression (Fig. 4A and B). Both ATF4 and ATF5 are known downstream targets of eIF2α (64) and have been found to be involved in the induction of FGF21 gene expression in muscle (37, 59). Thus, these data link increased cellular stress, collectively an increased ISR, to FGF21 secretion in SM, demonstrating its role as a novel homeostatic response to increased SM stress.
(Fig. 5C). These data clearly confirm that FGF21 acts as a myokine released from SM in response to an altered mitochondrial respiratory chain capacity and induced cellular stress response.

**FGF21 receptors in potential target tissues.** It has been suggested that FGF21 functions predominantly in a paracrine/autocrine manner because, so far, target tissues have also been the main tissues of FGF21 expression (35). However, the pronounced increase of circulating FGF21 in UCP1-Tg mice suggests possible endocrine actions. Therefore, we analyzed gene expression of FGF receptors FGFR1 and FGFR2 as well as the crucial coreceptor KLB in potential target organs. FGFR1 and FGFR2 (Fig. 6, A and B) showed opposite gene expression patterns throughout all tissues analyzed with FGFR1, showing very low expression levels in liver. In UCP1-Tg mice FGFR1 was downregulated, whereas FGFR2 was upregulated in liver. Gene expression of KLB was high in liver and SWAT and much lower in SM (Fig. 6D). However, on gene expression level, KLB was upregulated in liver. Gene expression of KLB in SM (Fig. 6C) was high in muscle, liver, and SWAT, and no KLB protein expression at all was detected in SM (Fig. 6D). However, on gene expression level, KLB was increased in all tissues investigated in UCP1-Tg mice, suggesting a possible feedforward effect of FGF21 on its own signaling pathway.

**Endocrine effects of skeletal muscle FGF21 on somatic growth and liver.** FGF21 has been shown to inhibit GH signaling characterized by decreased levels of IGF-I and increased plasma levels of GH, which result in a reduced body size of mice overexpressing FGF21 (29). Furthermore, the FGF21-overexpressing mice display a reduced bone mass (60, 63). UCP1-Tg mice show a similar phenotype of reduced body length and lean body mass (32, 34) that was confirmed again in UCP1-Tg mice FGFR1 was downregulated, whereas FGFR2 as well as phosphorylation of ACC1 and ACC2 (ACACB) as markers for the balance between fatty acid synthesis and fatty acid oxidation revealed no difference in liver of UCP1-Tg mice compared with WT (Fig. 7E). Therefore, we propose that, under standard diet conditions, liver substrate metabolism is not the main target of FGF21 secreted from uncoupled SM.

**SM-secreted FGF21 leads to “browning” of WAT.** WAT is an important target tissue of FGF21 action. Recently, FGF21 was found to induce the appearance of BAT-like adipocytes in WAT, with different depots showing various degrees of browning (19). UCP1-Tg mice show an increased glucose uptake (50) and an augmented lipid metabolism (32, 63) due to high variation; Fig. 7B). Additionally, they also showed a reduced bone mineral density (Fig. 7C). Most of the circulating IGF-I is produced in liver (62), and overall liver metabolism has been shown to be directly affected by FGF21 (18). Here, we found no differences in hepatic expression of genes related to lipid metabolism [peroxisome proliferator-activated receptor (PPAR)-γ and α, acetyl-CoA carboxylase (α-ACACA, ACC1), and carnitine palmitoyltransferase 1α] between UCP1-Tg and WT mice, but we found an induction of PPARγ coactivator-1α (PGC-1α) and phosphorylated pyruvate carboxykinase 1 (PEPCK1) gene expression (Fig. 7D), indicative of an increased gluconeogenesis rate and confirming previous findings with exogenous FGF21 application (17, 18). However, protein expression analysis of gluconeogenic markers (PEPCK1) and PGC-1α as well as phosphorylation of ACC1 and ACC2 (ACACB) as markers for the balance between fatty acid synthesis and fatty acid oxidation revealed no difference in liver of UCP1-Tg mice compared with WT (Fig. 7E). Therefore, we examined possible effects of FGF21 on different WAT depots, i.e., sWAT and epididymal WAT (eWAT) depots. Histology showed an altered morphology of both sWAT and eWAT from UCP1-Tg mice compared

**Fig. 6. FGF21 receptors in potential target tissues.** A–C: qPCR analysis of FGF receptor (FGFR)1 (A), FGFR2 (B), and β-Klotho (KLB; C) in SM, liver, and sWAT of WT and UCP1-Tg mice. D: representative immunoblots of KLB protein expression in muscle, liver, and sWAT (α-tubulin was used as a control). *P < 0.05, **P < 0.01, and ***P < 0.0001, significant differences between the genotypes; n = 6–10/group. Data are means ± SE.
with WT, with more small, multilocular adipocytes present within fat depots of UCP1-Tg mice compared with WT mice (Fig. 8A). This was accompanied by an increased expression of typical BAT marker genes such as UCP1, CIDEA, and PGC-1α in sWAT and in eWAT (Fig. 8B). Subsequent analysis of protein expression in sWAT from UCP1-Tg mice showed an increased expression of enzymes of the respiratory chain (indicative of increased mitochondrial content) and increased UCP1 protein compared with WT (Fig. 8C). Furthermore, we measured COX activity as a functional marker for maximal mitochondrial respiratory capacity. Indeed, UCP1-Tg mice showed a highly significant, more than sevenfold increase of COX activity compared with WT mice (Fig. 8D). To ensure that overexpression of the transgenic UCP1 is indeed skeletal muscle specific and that the increased UCP1 gene expression in sWAT represents the native form of UCP1, we designed a primer pair that binds to the native but not to the transgenic (muscle-specific) UCP1 mRNA. We detected no native UCP1 expression in skeletal muscle, a high expression in BAT, and an induction in sWAT of UCP1-Tg mice compared with WT (Fig. 8E). This confirms that the increased expression of UCP1 in WAT of UCP1-Tg mice is due to a browning effect and not to a “leaky” expression of transgenic UCP1. To investigate whether the increased UCP1 expression in WAT depots of UCP1-Tg mice is linked directly to Tg muscle-secreted FGF21, we performed an in vitro study, adding 20% blood serum of WT or UCP1-Tg mice to murine primary white adipocytes with and without FGF21 antibody. Strikingly, in primary WAT treated with serum of UCP1-Tg mice, UCP1 gene expression was increased compared with WT serum treatment. In contrast, when adding serum of UCP1-Tg mice with FGF21 antibody to the incubation medium, no differences in UCP1 RNA levels between WT and UCP1-Tg mice were observed (Fig. 8F). These data demonstrate a skeletal muscle FGF21-induced “browning” linked to an increased oxidative capacity of WAT in UCP1-Tg mice.

Increased futile cycle but no effect on GLUT1 or ERK in WAT of UCP1-Tg mice. Because FGF21 also plays an important role in lipid metabolism (28), we had a closer look at mRNA levels of proteins related to lipogenesis (FASN and ACACA) as well as lipolysis (adipose triglyceride lipase and HSL). Interestingly, lipogenic as well as lipolytic genes were increased in both WAT depots of UCP1-Tg mice compared with WT (Fig. 9A), confirming an increased futile cycle of lipid storage and release, as reported before (32). Moreover, HSL
was just recently identified as a direct FGF21 target in WAT (11). Performing further protein analysis in sWAT depots, we measured levels of the activating phosphorylation of HSL on Ser660, which was significantly upregulated in UCP1-Tg mice (Fig. 9B). MAP kinase ERK (p42/44 MAPK or ERK1/2) and the glucose transporter 1 (SLC2A1 or GLUT1) have been found to further intracellular downstream signaling targets of FGF21 (23). Measurement of phosphorylated ERK1/2 in sWAT and GLUT1 showed no differences between the genotypes (Fig. 9C). Altogether, this is in line with recent findings showing that FGF21 induces lipolysis in WAT without induction of ERK1/2 signaling pathways (11).

**DISCUSSION**

Here, we show evidence that the improved metabolic phenotype of UCP1-Tg mice is linked to an induction of endocrine-acting FGF21 expression and secretion from skeletal muscle. FGF21 is considered mainly as a starvation signal secreted by liver, although it is expressed in other tissues such as adipose tissue, SM, testis, and pancreas (3, 15, 21, 35, 51, 57). Until now, evidence for a possible contribution of muscle FGF21 secretion to circulating FGF21 was restricted to models of mitochondrial myopathy (37, 56, 59), and circulating FGF21 has been proposed as biomarker for primary muscle-manifest-
ing respiratory chain deficiencies in humans (56). We found a several-hundred-fold increase of FGF21 gene expression in SM of UCP1-Tg mice, resulting in five- to 10-fold elevated plasma levels compared with WT, which was considerably higher than levels detected in mice with a mitochondrial myopathy (59). In contrast to liver, there is no induction of FGF21 gene expression by 24-h fasting in SM (59), suggesting that the induction requires a more sustained stress insult than that provided by acute fasting and that different signaling pathways are involved in the secretion of FGF21 from liver and SM. Recently, we reported increased levels of lipid peroxidative damage and molecular stress markers (such as MAPK signaling and heat shock protein 25) linked to an upregulation of antioxidative defense (catalase and superoxide dismutase activity) in SM of UCP1-Tg mice (33). Here, we show additionally an activation of the p-eIF2α/ATF4 cascade that is part of the ISR, a gene-expression regulating program involved in substrate metabolism and cellular stress response (12). Recent studies in autophagy-deficient muscle have implicated this cascade in the induction of FGF21 gene expression due to mitochondrial dysfunction (37). Moreover, they showed that mitochondrial stressors also induce FGF21 through ATF4. Our results are supportive of this notion, and we propose the p-eIF2α/ATF4 cascade as a signaling pathway leading to the high FGF21 induction in SM of UCP1-Tg mice. Furthermore, increased mitochondrial ROS production does not seem to be necessary for FGF21 induction in SM, as evident from our in vitro treatment of C2C12 cells with different inhibitors of the respiratory chain.

Remarkably, UCP1-Tg mice showed no evidence for a myopathy despite decreased muscle mass and rather an activated than inhibited autophagic machinery, but without increased basal autophagy flux. In any case, FGF21 expression in muscle is not purely a consequence of pathological impairment of autophagy processes or dystrophic morphology in muscle. Moreover, our data support the notion that increased FGF21 levels reflect a protective response. Altogether, our data show that FGF21 secretion from muscle is related to altered mitochondrial function and metabolic stress.

It has been suggested that FGF21 functions predominantly in a paracrine/autocrine manner because, so far, target tissues have also been the main tissues of FGF21 expression (35). Our data suggest that FGF21 secreted from muscle acts as a true endocrine hormone, targeting tissues other than the secreting tissue. There seems to be no autocrine/paracrine effect of FGF21 itself on muscle, as evident from the absent protein expression of KLB, the coreceptor required for FGF21 action. This fits with the report that overall muscle gene expression levels were affected very little by overexpression of FGF21 in transgenic mice (63). The main target organs of FGF21 are muscle, as evident from the absent protein expression of KLB, the coreceptor required for FGF21 action. The main target organs of FGF21 are muscle, as evident from the absence of FGF21-induced effects on liver and muscle in UCP1-Tg mice. Moreover, fibronectin type III domain-containing 5/irisin, the only other myokine reported to induce WAT browning (6), is not upregulated in muscle of UCP1-Tg. Besides FGF21, IL-6 gene expression shows a much lower but significant upregula-
tion in SM of UCP1-Tg mice. This could be associated with increased muscle atrophy (24). Functional confirmation as well as elucidation of the molecular mechanism of IL-6 induction in muscle through mitochondrial uncoupling is a topic of future research.

In summary, our data suggest increased FGF21 secreted from muscle as a protective stress response and, furthermore, SM as an important endocrine regulator of whole body metabolism ultimately increasing longevity. These results open up new avenues of investigation that may help to resolve FGF21 function and regulation in humans, which is at present hardly understood.

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DISCLOSURES

There are no conflicts of interest, financial or otherwise, for any of the authors.

AUTHOR CONTRIBUTIONS

S. Keipert, M.O., and S. Klaus contributed to the conception and design of the research; S. Keipert, M.O., K.J., and F.I. performed the experiments; S. Keipert, M.O., K.J., and F.I. analyzed the data; S. Keipert, M.O., M.J., E.M.V.S., J.K., and S. Klaus interpreted the results of the experiments; S. Keipert and M.O. prepared the figures; S. Keipert, M.O., M.J., E.M.V.S., J.K., and S. Klaus edited and revised the manuscript; S. Keipert, M.O., M.J., and S. Klaus approved the final version of the manuscript; S. Klaus drafted the manuscript.

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