Inter-organellar and systemic responses to impaired mitochondrial matrix protein import in skeletal muscle

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Effective protein import from cytosol is critical for mitochondrial functions and metabolic regulation. We describe here the mammalian muscle-specific and systemic consequences to disrupted mitochondrial matrix protein import by targeted deletion of the mitochondrial HSP70 co-chaperone GRPEL1. Muscle-specific loss of GRPEL1 caused rapid muscle atrophy, accompanied by shut down of oxidative phosphorylation and mitochondrial fatty acid oxidation, and excessive triggering of proteotoxic stress responses. Transcriptome analysis identified new responders to mitochondrial protein import toxicity, such as the neurological disease-linked intermembrane space protein CHCHD10. Besides communication with ER and nucleus, we identified crosstalk of distressed mitochondria with peroxisomes, in particular the induction of peroxisomal Acyl-CoA oxidase 2 (ACOX2), which we propose as an ATF4-regulated peroxisomal marker of integrated stress response. Metabolic profiling indicated fatty acid enrichment in muscle, a shift in TCA cycle intermediates in serum and muscle, and dysregulated bile acids. Our results demonstrate the fundamental importance of GRPEL1 and provide a robust model for detecting mammalian inter-organellar and systemic responses to impaired mitochondrial matrix protein import and folding.
Mitochondria are key organelles in the regulation of cellular metabolism. The vital functions of mitochondria are largely performed by proteins imported into the organelle from cytosol. Nuclear-encoded mitochondrial proteome (~1500 proteins) is synthesized by cytosolic ribosomes and imported into mitochondria as precursor proteins. The protein import processes utilize targeting signals and specialized import pathways depending on which mitochondrial compartment the protein is to be targeted. Proteins directed to the mitochondrial matrix enter through the double membrane via the translocases of the outer membrane (TOM) and inner membrane (TIM23). Finally, the presequence translocase-associated motor (PAM) pulls the incoming proteins into the matrix. PAM has the mitochondrial heat shock protein 70 (mHSP70) at its core, and its ATPase cycle for substrate protein binding and release is regulated by nucleotide exchange factors (NEFs) and J-domain proteins. NEFs facilitate the ADP/ATP exchange and substrate release. In mammalian mitochondria, two bacterial GrpE-like NEFs, GRPEL1 and GRPEL2, have been identified, but the functional significance of the two is not clear. Both can regulate mHSP70 function and form hetero-oligomeric complexes in vitro. However, human variation data and studies in cultured cells have indicated an essential function only for GRPEL1, whereas GRPEL2 may have a stress-regulated role. Furthermore, GRPEL1, but not GRPEL2, is able to complement Mge1, the sole mitochondrial NEF in Saccharomyces cerevisiae.

Inability to import proteins into mitochondria results in cytosolic accumulation of mistargeted proteins, which activate proteotoxic stress response pathways. Studies in yeast have supported a protective role for the stress responses to counteract the impaired mitochondrial protein import by inhibiting global protein synthesis and activating the proteasome. Tissue-specific and systemic effects of compromised protein import in mammals have been studied less, however, other models of mitochondrial dysfunction have demonstrated that integrated stress response (ISR) is a common adaptation to mitochondrial defects. The core event of ISR is the phosphorylation of eIF2α, which leads to a decrease in global protein synthesis and the induction of selected genes that together promote cellular recovery. However, severe stress can drive ISR signaling toward cell death. Activated genes that together promote cellular recovery. However, severe stress can drive ISR signaling toward cell death.

Results and discussion
Loss of mitochondrial chaperone GRPEL1 in skeletal muscle of mice causes rapid muscular atrophy. To clarify the in vivo role of GRPEL1 in mammalian mitochondria, we intended to generate whole-body Grpel1 knockout mice. Heterozygous mice (Grpel1+/-) were viable with no apparent histological abnormalities (Supplementary Fig. 1a–d). However, Fl heterozygous crosses provided no homozygous Grpel1−/− pups, which were also lacking at embryonic day E8.5, indicating early developmental lethality (Fig. 1a and Supplementary Fig. 1e). These results confirm that GRPEL1 is essential in mammals.

To study tissue-specific consequences of GRPEL1 loss, we generated inducible skeletal muscle-specific Grpel1 knockout mice (Grpel1skm−/−) using tamoxifen (TMX)-inducible Cre recombinase and human α-skeletal actin (HAS) promoter (HAS-Cre mice) (Supplementary Fig. 2a). TMX-inductions were started for the mice at the age of five weeks. Unexpectedly, already within two weeks from the induction, Grpel1skm−/− mice started to lose weight rapidly unlike untreated littermates (Grpel1fl/fl) or TMX-treated wild type mice (Fig. 1b–d and Supplementary Fig. 2b, c). Indirect calorimetry, performed after two weeks of tamoxifen treatment, showed no significant differences in movement, oxygen consumption, respiratory exchange ratio or heat production between Grpel1skm−/− mice and controls (Supplementary Fig. 2d, e). Moreover, glucose tolerance and insulin sensitivity were not significantly altered in Grpel1skm−/− mice (Supplementary Fig. 3a, b). However, the phenotype advanced to the sacrificial point (30% weight loss, kyphosis, reduced movement) within five weeks from TMX-induction (Fig. 1e). At the end point, blood glucose was significantly reduced (Supplementary Fig. 3c). Skeletal muscles appeared atrophic, and grip strength was reduced (Fig. 1f, g). Detected by immunoblotting, the GRPEL1 protein level in quadriceps femoris (QF) muscle was reduced to about 40% of control level (Fig. 1h, i). Immunostaining of the QF muscle with GRPEL1 antibody further indicated reduced GRPEL1 protein in Grpel1skm−/− mice (Fig. 1j). Electron micrographs of QF showed an uneven width of sarcomeres and discontinuous Z-lines (Fig. 1k), and large mitolysosome-like structures in some fibres (Fig. 1k). Histological staining of QF with Hematoxylin & Eosin (HE) and Sirius Red in quadriceps femoris (QF) was reduced to about 40% of control level (Fig. 1h, i). Immunostaining of the QF muscle with GRPEL1 antibody further indicated reduced GRPEL1 protein in Grpel1skm−/− mice (Fig. 1j). Electron micrographs of QF showed an uneven width of sarcomeres and discontinuous Z-lines (Fig. 1k), and large mitolysosome-like structures in some fibres (Fig. 1k). Histological staining of QF with Hematoxylin & Eosin (HE) and Sirius Red in Grpel1skm−/− muscle revealed shrinkage of myofibers, and fibrosis, respectively (Fig. 1l–n). Histochemical activity staining of cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) showed some COX-negative muscle fibers, but none of those were SDH-positive ragged red fibers (Fig. 1l). These results indicated that the loss of GRPEL1 caused rapid atrophy and disorganization of skeletal muscle.

Loss of GRPEL1 leads to transcriptional repression of mitochondrial metabolic pathways. Next, we analyzed the transcriptome of Grpel1skm−/−. By RNA sequencing to identify transcriptional responses to GRPEL1 loss in muscle. Principal component analysis (PCA) indicated that the muscle of Grpel1skm−/− mice greatly differed from control muscle (Fig. 2a), with more than 6000 differentially expressed genes (FDR < 0.01) (Supplementary Data 1). These included transcriptional repression of the major mitochondrial metabolic pathways such as the TCA cycle, OXPHOS (RC-complex subunits), and mitochondrial fatty acid (FA) oxidation (Fig. 2b–d; Supplementary Data 2, 3; Supplementary Fig. 4). Mitochondrial DNA encoded transcripts were reduced (Fig. 2e), as well as mtDNA copy number (Fig. 2m). Most transcripts for mitochondrial ribosome subunits were downregulated (Fig. 2f). The shutdown of the key metabolic functions was supported by reduced respiration, as determined by respirometry, with lower OXPHOS complex I-IV activities (Fig. 2j, k), and reduced FA oxidation in mitochondria isolated from Grpel1skm−/− muscle (Fig. 2l). As an exception to the downregulation of OXPHOS transcripts, tissue-specific isoforms of COX subunits Cox6a1, Cox6a2 and Cox6b1 were increased. Similarly, Atg7fl/fl, which codes an inhibitor protein of ATP synthase (IF1) with the ability to conserve ATP at the expense of membrane potential when mitochondrial respiration is inhibited, was increased (Fig. 2c). Interestingly, a few mitochondrial pathways were...
also transcriptionally upregulated in Grpel1skm−/− muscle (Supplementary Data 3). Among those were some TOM (Tomm34, 22, 40, and 20), but not TIM subunits (Fig. 2g). PAM complex transcripts Hspa9 (mtHSP70) and Grpel2, the paralog of Grpel1, were also increased as well as mitochondrial chaperones Dnaja3 (HSP40) and Hspe1 (HSP10). The expression of some mitochondrial proteases, such as matrix proteases Clpp and Lonp1, and inner membrane or intermembrane space (IMS) proteases Afg3l2, Immp2l and Htra2, was increased (Fig. 2h). These findings may suggest that GRPEL1 loss activates mitochondrial chaperones and proteases to balance impaired protein folding within mitochondria, although this cannot be concluded from transcriptional data. We also observed that the expression of amyotrophic lateral sclerosis (ALS)-linked gene Chchd10 was significantly increased in Grpel1skm−/− muscle, which
Inducible loss of mitochondrial co-chaperone GrpEL1 in skeletal muscle of mice causes rapid muscular atrophy, retarded growth and shortened lifespan. a A schematic for the study of whole-body and skeletal muscle-specific knockouts of GrpEL1 (Created with BioRender.com). b Timeline of Cre-activation in mice by tamoxifen (TMX) injections. c Representative images of GrpEL1flkox/lox (right) 4 weeks after tamoxifen injection and the control littermate GrpEL1flk/lox (left). d Body weight curve of male mice, starting from TMX injection. Four different groups of mice are shown: GrpEL1flk−/− are the inducible skeletal muscle-specific GrpEL1 knockout mice, GrpEL1flklox/lox_Cre are the floxed Cre mice without tamoxifen, GrpEL1flklox/TMX are the floxed mice without Cre but with tamoxifen, and WT_Cre_TMX are wild type Cre mice with tamoxifen. (n = 10–12 per genotype). Tukey’s multiple comparisons test. **p ≤ 0.0001. e Survival curve of GrpEL1flklox−/− mice analysed with Gehan-Breslow-Wilcoxon test. (n = 13 per genotype). f Representative images of quadriceps of GrpEL1flk−/− (right) and control littermate GrpEL1flklox/lox (left) at sacriﬁcation point. g Grip strength of mice immediately before tamoxifen injection and at end point (n = 5–7 per genotype). h Immunoblot showing reduced GrpEL1 level in skeletal muscle of GrpEL1flk−/−, in comparison to GrpEL1flklox/lox and wild type (WT) mice. Vinculin is shown as loading control. i Quantification of GrpEL1 protein level in GrpEL1flk−/− muscle from immunoblot against Vinculin (n = 3 per genotype). j Representative immunohistochemistry images of quadriceps femoris (QF) from control and knockout mice with GrpEL1 and SDHA antibodies. Scale bar is 100 µm. k Representative electron microscopic images of QF muscle, showing the uneven width of sarcomere and discontinuous Z-line in knockout (left, arrows) and large vacuole structures identiﬁed in some ﬁbers (right). l Representative Hematoxylin/Eosin, COX/SDH, and Sirius Red (ﬁbrosis) staining images of QF muscle. m Quantification of cross-sectional area of myofibril in QF muscle (n = 30 representative myofibrils from 3 mice per genotype). n Quantification of ﬁbrotic area of QF muscle (n = 3 mice per genotype).

Transcriptional alterations indicative of inter-organellar communication in GrpEL1 knockout muscle. We then focused on transcriptome alterations affecting pathways outside mitochondria to address inter-organellar communication as a response to impaired mitochondrial protein import. The expression of some, but not all, proteasome subunits were increased (Fig. 3a). ER stress and ISR were among the most signiﬁcantly induced pathways, supporting that GRPEL1 loss was leading to mistargeted proteins along with other stress response genes such as Prdx3, Prdx5, and proteotoxic stress (Fig. 3b, c). Secreted ISR cytokine genes Fgf21 and Gdf15, which have virtually no expression in healthy muscle, were among the most highly induced transcripts, along with other stress response genes such as Ddit3 (CHOP), Mthfd2 and Psat1. These stress responses were accompanied by increased eIF2α phosphorylation (Fig. 3j–l), and increased expression levels of Atf4 (2.3-fold), Atf5 (5.0-fold) and Atf6 (3.8-fold), the latter being the effector of ATF6 from ER stress.23 We also observed a striking elevation in transcripts of cytosolic ribosome some subunits and cytosolic tRNA synthetases (Fig. 3d, e). This contrasts with attenuation of protein synthesis in acute proteotoxic stress and suggests that the GrpEL1flk−/− muscle was in prolonged stress leading to enhanced protein synthesis.13,24–26 Indeed, phosphorylation of ribosomal protein S6 (p-S6), a downstream target of mTORC, was increased in GrpEL1flk−/− muscle (Fig. 3j, m, n). Stress-induced increase in protein synthesis has been shown to lead to oxidative stress and cell death.25 In GrpEL1flk−/− muscle many antioxidant defense genes such as Cat (catalase) were upregulated, but mitochondrial Sod2, Prdx3, Prdx5, and glutaredoxins were downregulated (Fig. 3f). Transcripts related to autophagy and apoptosis were also increased (Fig. 3g). Recent study has shown that ISR-linked pathology in mitochondrial myopathy is associated with stalled autophagy in the advanced stage when mTORC becomes activated.27 Accordingly, we observed a clear increase in p62/SQSTM1 levels, and a shift in LC3BI/II ratio in GrpEL1flk−/− muscle by Western blot (Fig. 3j).

ACOX2 is a peroxisomal ISR marker. Interestingly, we also identiﬁed increased gene expression on the peroxisomal beta-oxidation pathway (Fig. 3h). In particular, the peroxisomal Acyl-CoA oxidase 2 (Acox2) showed high induction (Figs. 3h and 4a), suggesting that peroxisomal metabolism may be activated in muscle in response to mitochondrial shutdown. ACOX2 is involved in the beta-oxidation of bile acid intermediates, resulting in the CoA esters of cholic and chenodeoxycholic acid.22 Reminiscent of key ISR markers such as Fg21 and Gdf15, Acox2 is barely expressed in normal skeletal muscle, but had high expression in GrpEL1flk−/− muscle. Acox2 expression has not been previously linked to ISR, but we noticed that it was also highly expressed in the Dars2 knockout mice, which have highly induced ISR owing to impaired mitochondrial translation.13 According to ChIP-seq data, ATF4 may regulate the expression of Acox2.23 We thus suspected that Acox2 could be a peroxisomal marker of ISR. To test whether its elevation was speciﬁc to muscle, we studied ACOX2 expression in human skin ﬁbroblasts treated with actinonin, a known ISR inducer that blocks mitochondrial protein synthesis28 and induces ATF4 and ATF5 expression. Intriguingly, actinonin treatment induced ACOX2 expression (Fig. 4b), suggesting that it has a yet uncharacterized role in proteotoxic stress. Next, we tested if ACOX2 induction was regulated by ATF4 or ATF5 using siRNA mediated knockdown of these transcription factors. The results showed that actinomin-induced expression of ACOX2 was repressed by ATF4 siRNA but not by ATF5 siRNA (Fig. 4c, d), demonstrating that ACOX2 is an ATF4-regulated ISR target gene. Taken together, these results indicate that GRPEL1 loss in skeletal muscle activates crosstalk with the nucleus, ER, proteasome and the peroxisomes. However, the activation of the stress responses does not rescue from pro-apoptotic signaling and muscle atrophy (Fig. 3i).
FA, and ketone bodies) increased in muscle, suggesting a metabolic shift (Fig. 5b, c, d). A combined analysis revealed a possible metabolite exchange between serum and muscle (Fig. 5b), as some TCA cycle metabolites appeared depleted in serum and increased in muscle (Fig. 5e), although any exchange cannot be concluded without more detailed studies. Altered amino acid metabolism indicated protein breakdown in muscle (Fig. 5c). Creatinine and carnosine were depleted in both serum and muscle, consistent with reduced muscle mass (Fig. 5f, h). Elevation in nucleotide metabolism was detected in muscle (Fig. 5c, h), as previously reported in ISR-linked mitochondrial myopathy. Adenylate–energy charge ratio in muscle was lower (Fig. 5i), indicating poor energy status. Lower ratio of reduced (GSH) to oxidized (GSSG) glutathione reflected higher oxidative stress.
Fig. 2 Intramitochondrial response to GrpEL loss in skeletal muscle. a Principal component analysis (PCA) for RNA sequencing data from QF of GrpEL<sup>fix−/−</sup> and control mice (n = 4 per genotype). b-h Volcano plots showing the changes in gene expression in different mitochondrial pathways. i Graphical representation of the altered mitochondrial pathways in GrpEL<sup>fix−/−</sup> muscle. Red color (arrows and text) indicates upregulation whereas the blue color (arrows and text) indicates downregulation (Created with BioRender.com). j Enzymatic activity of mitochondrial respiratory chain complexes, in mitochondria isolated from QF muscle, and normalized to control (n = 4 per genotype). k Ci-coupled, Ci− Ci-coupled and uncoupled (with FCCP) respiration in QF muscle homogenates (n = 4 per genotype). l Quantification of fatty acid oxidation capacity of mitochondria, isolated from QF muscle, and normalized to control (n = 3 per genotype). m Quantification of mtDNA copy number in QF muscle of GrpEL<sup>fix−/−</sup> and control mice (n = 4 per genotype). n Immunoblot of total protein lysates from QF muscle with CHCHD10 and HSP60 antibodies. o Representative images of immunohistochemistry staining of QF muscle with CHCHD10 antibody. Scale bar is 100 μm.

adaptation before recordings. The body composition, i.e., fat and lean mass, of the mice was estimated with Bruker minispec LF50 Body Composition Analyzer.

Grip strength. Muscle strength was determined with a grip strength meter. The mice were placed over the grid, gently pulled by its tail, and allowed to grasp the mesh grid by its fore and hind limbs. Six consecutive measurements were performed with a minute interval for each mouse. The mean maximal grip strength was recorded and then normalized to the body weight of individual mouse.

Glucose and insulin tolerance tests. A 6 h fasting period was set for the glucose tolerance test by removing food from the cages. The basal blood glucose was measured by tail vein puncture and then the mice were given glucose (1 g/kg of body weight) by IP injection. The blood glucose levels were measured at 15, 30, 60, 90, and 120 min with a glucometer (Accu-Chek Aviva Glucose Meter, Roche). For the insulin sensitivity test, basal blood glucose level was measured by puncturing the tail vein. Then the mice were given intraperitoneal insulin injection (0.8 U/kg of body weight) (Novolin R, Novo Nordisk) and the blood glucose was measured at 15, 30, 60, 90, and 120 min after the injection.

Histology. After the tissue samples were collected, they were fixed in 10% buffered formalin for one to three days at 4 °C. Tissues were then processed and paraffinized using Sakura Tissue-Tek VIP processor and next day embedded in paraffin blocks. For Hematoxylin and Eosin (HE) staining<sup>[45,46]</sup>, the tissue blocks were sectioned and stained with Hematoxylin for 1 min and with Eosin for 2 min, with washes in between the stains. Dehydration was done with absolute EtOH, cleared with xylene. The sections were then mounted with Pertex mounting medium (Histolab 00811). For the analysis of collagenization in skeletal muscle, paraffin samples were stained with Picrosirius Red staining. Samples were first de-paraffinized with xylene and rehydrated using descending EtOH series, cleared with xylene and mounted with Pertex mounting medium (Histolab 00811). For the stainings of cytokerin and vimentin, paraffin sections were deparaffinized, rehydrated and re-embedded in OCT medium. The sections were then deparaffinized and rehydrated using 2-methylbutane bath in liquid nitrogen. After freezing tissues were embedded with OCT Embedding Medium and kept at −20 °C for 90 min. Next day the tissue sections were cut into 10 μm sections and mounted on glass slides. The slides were then stained with CD31 antibody to stain blood vessels and cleared with xylene and mounted with Pertex mounting medium (Histolab 00811). All the stained sections were imaged with Axioplan 2 Universal Microscope (Zeiss).

Immunohistochemistry. Immunohistochemistry was done both from formalin-fixed paraffin embedded samples and frozen samples. Formalin-fixed paraffin embedded samples were first deparaffinized and rehydrated using xylene and descending EtOH series. Antigen retrieval was done by heating the samples 15 min in pressure cooker at 95 °C in 10 mM EDTA or 0.1 M citrate buffer, pH 6. Frozen samples were first fixed in 20 min at room temperature and rinsed with 0.1% PBS-Triton X-100. X-100 Aqueous. The primary antibodies were diluted in Dako antibody diluent (Agilent S3022) and incubated overnight at + 4 °C. The following primary antibodies were used: anti-GrpEL1 (NB1-P54665, Novus Biologicals), mouse anti-SDHA (ab14715, Abcam), rabbit anti-CHCHD10 (HPA003440, Sigma). Next day samples were washed three times with 0.1% PBS-Triton X-100 and secondary antibodies were diluted in Dako antibody diluent and incubated 30 min at RT. The secondary antibodies were washed with PBS-Triton X-100 for 5 min and mounted with Vectorshield mounting media with DAPI (Vector laboratories H-1200). Images were taken with Leica DM5000B wide field microscope.
Fig. 3 Organelle crosstalk in response to GrpE1 loss in skeletal muscle. a–h Volcano plots for different pathways indicating changes in gene expression due to loss of GrpE1 in skeletal muscle. i Graphical representation of crosstalk between dysfunctional mitochondrial, due to loss of GrpE1, with other cellular organelles. Red color (arrows) indicates upregulation whereas the blue color (arrows) indicates downregulation (Created with BioRender.com).

j Immunoblot of total protein lysates from QF muscle of GrpE1<sup>skm−/−</sup>, GrpE1<sup>flx/flx</sup> and WT mice. k–n Quantification of p-EIF2α level, p-EIF2α/total EIF2α ratio, p-S6 level and p-S6/total S6 ratio in skeletal muscle of GrpE1<sup>skm−/−</sup>, GrpE1<sup>flx/flx</sup> and WT mice. Vinculin was used as the loading control (n = 3 per genotype).
after treatment with actinonin, compared with vehicle (ethanol).

**Mitochondrial DNA copy number analysis.** Total DNA was extracted from snap frozen QF muscle of Grpel1skm−/− mice by phenol-chloroform extraction method and quantified using Spectrophotometer (DeNovix, DS-11 Series). 30 ng of isolated DNA was used as template. The mitochondrial DNA was quantified by amplifying the mitochondrial 12 S rRNA gene and normalized to nuclear RBM15 gene. Primers used were: 12S_Forward: AGGAGCCTGTTCTATAATCGATAAA, 12 S_Reverse: GATGGCGGTATATAGGCCGAA, RBM15_Forward: GGACAGTTTTCTTGCCCAAC and RBM15_Reverse: AGTCTGGCCCTGAGACAT.

**High-resolution respirometry.** Total tissue lysates were used in Oroboros for respiration measurement. Tissue lysates in MiR05 (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 10 mM KH2PO4, 20 mM HEPES, and 110 mM sucrose, pH 7.1) were taken to the chamber. We measured the response in oxygen flux for CI-coupled respiration after adding malate (final concentration 2 mM), pyruvate (10 mM), glutamate (20 mM), ADP (5 mM), and cytochrome C subsequently into the chamber. The CI + CII coupled respiration was measured with the addition of succinate (10 mM). Subsequent addition of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (2 mM) provided maximal respiration (CI + CIII uncoupled respiration) of mitochondria. Basal respiration was measured by inhibiting the reaction with rotenone (0.1 μM) and antimycin (2.5 μM). Total protein concentration of tissue lysate was used for normalization.

**Respiratory chain enzyme activity measurements.** Isolated mitochondria were used for measuring RC complex activity. We isolated the mitochondria from skeletal muscle and followed the protocol of Spinazzi et al. 201232 for the measurement of enzymatic activity of CI-CIII. Complex IV activity was measured using Oroboros from flux response to ascorbate (10 μM) and TMPD (2.5 μM) after inhibiting CIII with antimycin A. The complete reaction was compromised by adding Azide (50 μM) to the chamber. We normalized the enzyme activities to total protein concentrations.

**Immunoblotting.** The tissue samples were snap frozen in liquid nitrogen and stored in −80 °C. About 20 mg of snap frozen tissues were taken and homogenized in RIPA buffer (Cell signaling technology) containing Halt™ Protease-inhibitor Cocktail (ThermoFisher) with Fast Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). Protein concentrations were quantified with Bradford method (Bio-Rad). 20 μg of protein per sample were loaded into 10% stain free polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes using transfer-blot turbo transfer system (Bio-Rad). The membranes were then blocked with 5% milk in 1X TBS-T to avoid nonspecific binding. The

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**Fig. 4 ACOX2 is a peroxisomal marker for ISR.** a Quantification of Acox2 mRNA expression in QF muscle of Grpel1skm−/− mice (n = 4 per genotype). b Timeline showing the induction of ACOX2, ATF4 and ATF5 mRNA expression in human fibroblasts (C1 and C2 are independent control fibroblast lines) after treatment with actinonin, compared with vehicle (ethanol). c Quantification of ATF4 and ACOX2 mRNA expression after siRNA-mediated knockdown of ATF4 followed by 24 h of actinonin treatment (n = 3 per treatment). d Quantification of ATF5 and ACOX2 mRNA expression after siRNA-mediated knockdown of ATF5 followed by 24 h of actinonin treatment (n = 3 per treatment).
primary antibodies were prepared in 1% BSA/TBS-T with the concentrations recommended by the manufacturers. After subsequent washing, membranes were incubated with following primary antibodies overnight at 4 °C: rabbit anti-GRPEL1 (NB1-54665, Novus Biologicals), goat anti-HSP60 (sc-1052, Santa Cruz Biotechnology), rabbit anti-GAPDH (14C10, Cell Signaling), rabbit anti-phospho-S6 ribosomal protein (Ser 240/244) (5364, Cell Signaling), rabbit anti-LC3B (NB600-1384, Novus Biologicals), mouse anti-vinculin (V9264, Sigma) and mouse anti-p62 (2C11, Abnova). Membranes were washed and then incubated with respective HRP conjugated secondary antibodies for 1 h in room temperature. The Clarity Western ECL Substrate (BioRad) was used to develop protein band signals, detected by Chemidoc XRS (V9264, Sigma) and mouse anti-p62 (2C11, Abnova). Membranes were washed and then incubated with respective HRP conjugated secondary antibodies for 1 h in room temperature. The Clarity Western ECL Substrate (BioRad) was used to detect the protein band signals, detected by Chemidoc XRS (V9264, Sigma) and mouse anti-p62 (2C11, Abnova).
Mitochondrial fatty acid oxidation measurement
The QC muscle samples (100 mg) were homogenized in STE buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA) and mitochondria were isolated. The isolated mitochondrial fraction was incubated in a reaction buffer (14C-radiolabeled oleic acid (NEC317250UC-Perkin Elmer), 2 mM ATP, and 0.5 mM dithiothreitol) for 40 min at 37 °C. The CO2 released from fatty acid oxidation were trapped in 1 M NaOH-soaked filter paper and measured for acid-soluble metabolites using a scintillation count.

Transmission electron microscopy
The tissue samples were fixed in 2.5% glutaraldehyde for 2 h and stored in 2% PFA at 4 °C. Tissue samples were embedded into blocks, cut into thin sections and stained according to the standard procedure of the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki, Finland. Plus transmission electron microscope was used for imaging and Imaged software for quantifications.

RNA isolation and sequencing
Total RNA was isolated from snap frozen QC with mirVana RNA extraction kit (Qiagen). The tissue samples were homogenized using Fast Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). The NEBNext Ultra II Directional DNA Library Prep Kit for Illumina was used to generate sequencing libraries. From 1 μg of total RNAs, the polyA tail was removed and the reverse complement of Annexin A5 (ATGCTCCCCGGGCTGTAT) was ligated using T4 RNA Ligase (NEB), 2 mM ATP, and 0.5 mM dithiothreitol for 40 min at 37 °C. The amplified library was then purified using AMPure XP beads and its quality was assessed by TapeStation (Agilent High Sensitivity D5000 assay) and library quantity by Qubit. The prepared libraries were then sequenced with Illumina NextSeq500-system (Mid Output 2 x 75 bp).

Raw data (bcl-files) was demultiplexed with bcl2fastq2 (v2.20.0.422; Illumina), filtered read pairs filtering using Fast Prep w-24 Lysing Matrix D (MP Biomedical) and Precellys w-24 (Bertin Technologies). The tissue samples were sequenced using NextSeq500-system (Mid Output 2 × 75 bp).

Metabolites profiling
Mice were terminally anaesthetized with Pentobarbital (100 mg/kg) and blood was collected by heart puncture. The blood collection tubes were kept in ice for 15 min with serum separator and clot activator (MiniCollect Tube, Breiner-bion-one, 450533). The tubes were centrifuged at 5000RPM for 10 min and serum was transferred to a new tube and was kept at −80 °C until use. QC samples were collected from the same set of anaesthetized mice and snap frozen and kept at −80 °C until use. The metabolites were extracted from 20 mg skeletal muscle (QF) tissue using 2 ml Preseqli homoegnation tube (Bertin Technologies, with 2.8 mm ceramic (zirconium oxide) beads with 400 μl of cold extraction solvent (Acetonitrile:Methanol:H2O; 40:40:20). Subsequently, the samples were homogenized using tissue homogenizer (Bertin Technologies,) for 3 min (30 sec at 6000 rpm with 400 μl at 4 °C). Furthermore, the homoegnized samples were centrifuged at 14000 rpm at 4 °C for 10 min. For mouse serum, metabolites were extracted from 50 μl serum with 400 μl of cold extraction solvent (ACN:MEOH:HO2; 40:40:20). After centrifugation the supernatant was loaded into Phree Phospholipid removal 96-well plate (RE-S133-TGB, Phenomenex) and the sheath gas: 25 arbitrary units (AU), and the auxiliary gas: 15 AU, sweep gas flow 0, Capillary temperature: 275 °C, S-lens RF level: 50.0, Instrument control was operated with the Xcalibur 4.1.31.19 software (Thermo Fischer Scientific).

In the data processing, the final peak integration was done with the TraceFinder 4.1 software (Thermo Fischer Scientific) using confirmed retention times of 462 inch-mass spectrometry library developed using library kit MSMLS-1EA (Merck). For further data analysis, the peak area data was exported as excel file. The data quality was monitored throughout the run using pooled sample as Quality Control (QC) prepared by pooling 5 μl from each suspended samples and interspersed throughout the run as every 10th sample. After integration of QC sample data with TraceFinder 4, the detected metabolites were checked for carryover effect and calculated against the mean QC area and acceptance limit was set to ≤ 5% for each metabolite. Background % noise was calculated with respect to first blank against the mean QC area and acceptance limit was set to ≤ 5% for each metabolite.

Metabolite data analysis
The data was analyzed with Metaboanalyst 5.0, and the missing variables were imputed using KNNVAR. The values were normalised to the tissue weight, Log10 transformed and auto scaled. Following statistical analysis were performed: Univariate analysis methods: t-tests (FDR-adjusted p-value ≤ 0.05) metabolites were significantly altered in Grpel1+/− mice compared to wild-type mice.
threshold 0.05, volcano plot (FC > 2; t-tests p-value < 0.05); Multivariate analysis methods: principal component analysis (PCA) and clustering analysis; heatmap (distance measure using euclidean, and clustering algorithm using ward.D). The differential expression was analyzed with limFit using contrasts to compare knockout to wild type within each sample type. The differentially expressed metabolites (FDR < 0.01) were visualized with a volcano plot generated with ggplot2 (v.3.3.5). PCA was calculated with prcomp using the voom-normalized expression values. The enrichment analyses were performed for significantly altered metabolites (P < 0.05) using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/) against KEGG database. To visualize pathway changes, bubble maps of enriched metabolites (significantly different P < 0.05) with mean log fold change (FC) were generated. Individual pathways or metabolites are plotted with mean log FC and standard deviation.

**Statistics and reproducibility.** The data are presented as mean ± SD, unless stated otherwise. The statistical analyses were performed with student’s t-test, and one-way or two-way ANOVA or as indicated in the figure legends. P-values less than 0.05 were considered significant. The significant differences between data or groups are indicated in the figures by **P < 0.05, ***P < 0.01, ****P < 0.001. Statistics was performed with GraphPad Prism 9.2.0. The data analysis for RNA sequencing and metabolite profiling are explained in the respective method sections.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The results from the gene expression analysis together with the raw sequences have been deposited to GEO, accession GSE195840. The list of differentially expressed genes is in Supplementary Data 1.xlsx. The pathway enrichments of the transcriptome data are in Supplementary Data 2 file. Transcriptome data for MitoCarta3.0 genes is in Supplementary Data 3 file. Metabolite profiling data from serum and muscle are in Supplementary Data 4 file. Source data underlying the graphs are provided in the Supplementary Data 5 file. Images of uncropped WB raw data are provided in Supplementary Figs. 5–7.

Received: 22 March 2022; Accepted: 25 September 2022;
Published online: 05 October 2022

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**Acknowledgements**

We thank Rätta Lehtinen and Pooja Manjunath for technical assistance. Riikka Kivelä and Nebeuyu Gizaw are acknowledged for providing the Cre mice. Biomedicum Functional Genomics Unit, Electron Microscopy Unit of the Institute of Biotecnology,
Finnish Centre for Laboratory Animal Pathology (FCLAP) and Biocenter Oulu Transgenic Core Facility are acknowledged for their services and collaborations. FIMM Metabolomics Unit was supported by HiLIFE and Biocenter Finland. This work was supported by European Research Council (grant number 637458), Academy of Finland, and Biomedicum Helsinki Foundation.

**Author contributions**

N.N., J.R., and H.T. conceptualized and planned the project. N.N., J.R., S.H. and B.H. carried out the experiments and analyzed the data. J.K. analyzed transcriptome and metabolite data. V.K. performed the mouse genotyping and histology. Y.Y. assisted in CLAMS measurements, and glucose and insulin tolerance tests. A.N. provided the metabolite profiling data. N.N., J.R., and H.T. wrote the manuscript. H.T. supervised the project. All authors reviewed the manuscript and approved it for submission.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s42003-022-04034-z.

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**Peer review information** Communications Biology thanks the anonymous reviewers for their contribution to the peer review of this work. Primary Handling Editors: Christopher Hine and Manuel Breuer.

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