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Store-Operated Ca^{2+} Entry Controls Induction of Lipolysis and the Transcriptional Reprogramming to Lipid Metabolism.

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Store-operated Ca^{2+} entry (SOCE) regulates lipid metabolism

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Abstract

A role for Ca^{2+} signaling in the regulation of lipid metabolism has been suggested, but the Ca^{2+} channels involved in this process remain elusive. Store-operated Ca^{2+} entry (SOCE) is a ubiquitous Ca^{2+} influx pathway that is regulated by the endoplasmic reticulum (ER) Ca^{2+} sensors stromal interaction molecule (STIM) 1 and 2, and the plasma membrane Ca^{2+} channel ORAI1. SOCE-deficient mice with abolished SOCE accumulate pathological amounts of lipid droplets in the liver, heart and skeletal muscle. Cells from patients with loss-of-function mutations in *STIM1* and *ORAI1* show lipid droplet accumulation, suggesting that the role of SOCE in lipid metabolism is conserved in humans. Functionally, SOCE contributes to regulating levels and usage of intracellular lipids by modulating lipolysis, lipophagy and mitochondrial function. Mechanistically, SOCE controls expression of PGC-1α and PPARα, master regulators of lipid metabolism. Our data demonstrate that SOCE is a crucial regulator of lipid metabolism.

Introduction

Previous studies have suggested a role for Ca^{2+} in controlling cell metabolism[1]. Intracellular Ca^{2+} has been found to regulate both the activity and expression of key metabolic enzymes. Allosteric regulation of metabolic enzymes by Ca^{2+} controls metabolic pathways, such as the tricarboxylic acid (TCA) cycle in which activity of the rate-limiting enzymes depends on Ca^{2+}binding[2-4]. Transcriptional control of metabolism by Ca^{2+} is exerted indirectly via Ca^{2+} dependent kinases and phosphatases, such as calmodulin-regulated kinases (CAMK) and calcineurin that control expression of the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α)[5-7]. At the organismal level, alteration of intracellular Ca^{2+} signaling has been linked to obesity[8] and related comorbidities[9], and dietary Ca^{2+} consumption to protection from obesity[10,11]. However, little is known about the Ca^{2+} channels involved in these regulatory processes and the identification of these channels is of critical importance in order to therapeutically modulate Ca^{2+} responsive metabolic pathways.

Store-operated Ca^{2+} entry (SOCE) is mediated by two families of proteins: ORAI proteins in the plasma membrane form the Ca^{2+} release-activated Ca^{2+} (CRAC) channel that conducts Ca^{2+} influx from the extracellular space [12-14], and stromal interaction molecules (STIM) 1 and 2 that are located in the membrane of the endoplasmic reticulum (ER) and bind to ORAI proteins resulting in the opening of CRAC channels[15,16]. Ca^{2+} influx via ORAI channels is called store-operated as STIM proteins are activated following a reduction in the ER Ca^{2+} concentration in response to stimulation of cell surface receptors that induce production of inositol 1,4,5-trisphosphate (IP_3) and opening of Ca^{2+} release channels in the ER such as the IP_3 receptor (IP_3R)[17]. The subsequent reduction in the ER Ca^{2+} concentration results in the dissociation of Ca^{2+} from an ER luminal EF-hand domain in the ~~N~~ N-terminus of STIM1[15,16] and conformational changes in the cytoplasmic tail of STIM1 that allow it to bind to ORAI[18,19].The latter are tetraspanning plasma membrane proteins that form hexameric complexes and constitute the Ca^{2+} permeant pore of the CRAC channel[12-14,20]. Patients with loss-of-function mutations in *ORAI1*, the best

characterized member of the ORAI family, or *STIM1* suffer from an autosomal-recessive disease syndrome named CRAC channelopathy that is characterized by severe immunodeficiency, muscular hypotonia and anhidrotic ectodermal dysplasia[19,21]. The cellular mechanisms underlying disease pathogenesis in different tissues are incompletely understood.

Here we identify SOCE as a critical regulator of lipid metabolism in mouse and human cells. We find that ORAI1- or STIM1/STIM2-deficient mice that lack SOCE accumulate pathological amounts of lipid droplets (LD) in their skeletal and heart muscles as well as their liver. LD accumulation is at least partially cell-intrinsic since isolated fibroblasts from human patients with loss-of-function mutations in *STIM1* or *ORAI1* also show increased LD levels. We identify CRAC channels to be responsive to extracellular fatty acids. This is functionally relevant since in the absence of SOCE cells are impaired in conventional lipolysis, and rely on lipophagy to protect themselves from lipotoxicity. We find that SOCE is crucial for the basal expression and fatty acid challenge-mediated induction of peroxisome proliferator-activated receptor α (PPARα) and PGC-1α genes, and for the expression of downstream genes involved in fatty-acid metabolism. Based on these results, we identify CRAC channels and SOCE as critical regulators of lipid metabolism.

Results

To investigate the role of SOCE in the regulation of lipid metabolism we used *Orai1*^{R93W} knock-in mice that express a non-functional ORAI1 channel protein[22], which abolishes SOCE in all tissues and is equivalent to the human ORAI1-R91W mutation found in a patient with CRAC channelopathy[12]. As reported previously, heart and skeletal muscle of *Orai1*^{R93W} mice lack histological and ultrastructural abnormalities except for a small fraction of skeletal myofibers containing markedly swollen mitochondria with abnormal cristae structure[22]. However, heart and skeletal muscle accumulate abnormal amounts of LDs as is apparent on transmission electron microscopy (TEM) images and by Oil Red O staining of muscle biopsies (**Fig.1a, b**). Because *Orai1*^{R93W}mice are perinatally lethal, we established a tamoxifen-inducible mouse model in which *Stim1* and *Stim2* genes can be deleted in all tissues of adult animals. Treatment of *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$*UBC-ER*^{T2}*-Cre* mice with tamoxifen resulted in rapid development of muscular dystonia, myoclonus-like symptoms (**Supplementary Videos 1-3**) and the death of mice within 5-10 days. Histologies of their heart muscle, skeletal muscle and liver revealed the accumulation of pathological amounts of LDs when compared to their Cre-negative littermate controls injected with tamoxifen (**Fig.1c**). A muscle biopsy from a human patient with G98R loss-of-function mutation in ORAI1 that abolishes SOCE showed LD deposition when investigated by TEM (**Fig.1d**). LD accumulation in the absence of Ca^{2+} influx is confirmed by the analysis of fibroblasts from patients with loss-of-function mutations in *ORAI1* or *STIM1* that abolish SOCE (**Fig.1e**). SOCE-deficient patient fibroblasts accumulated significantly higher amounts of LDs compared to healthy donors when cultured in high-glucose media (**Fig. 1f,g**) and when challenged with oleic acid (**Fig. 1h,i**). Collectively these findings suggest that SOCE has a conserved role in controlling lipid metabolism in mice and humans both in vivo and vitro.

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Lipid catabolism in cells depends on mitochondria, and a decline in mitochondrial function is linked to triglyceride accumulation in the muscle and liver[23]. Intracellular Ca^{2+} has been shown to regulate mitochondrial function[24]. To test if a specific role of SOCE in mitochondrial function could account for the higher lipid content in SOCE deficient cells, we analyzed fibroblasts from patients with loss-of-function mutations in *ORAI1* or *STIM1* that abolish SOCE. The mitochondrial volume was decreased in SOCE-deficient cells when measured using MitoView Green, a membrane potential-insensitive mitochondrial dye (**Fig. 2a,b**) and by TEM (**Supplementary Fig. 1**). By contrast, the mitochondrial DNA copy numbers assessed by quantitative PCR was normal in patient fibroblasts (**Fig.2c**) and skeletal muscle biopsies of three SOCE-deficient patients (data not shown), which is consistent with normal expression of transcription factors responsible for mitochondrial genome replication, *Transcription Factor A Mitochondrial* (*TFAM*) and *Nuclear Respiratory Factor (NRF) 1* and *2* (**Fig.2d**)[25]. However, the expression of components of the electron transport chain (ETC) complexes CI, CIII and CIV in mitochondria, which are critical for oxidative phosphorylation and ATP production, was reduced in SOCE-deficient fibroblasts, whereas expression of complex CII, which is entirely encoded by the nuclear DNA, and CV was normal (**Fig. 2e. Supplementary Fig. 2b**). Further analysis of isolated mitochondria by BN-PAGE confirmed reduced expression of complexes CI and CIV and of supercomplex CICIII2 in patient fibroblasts (**Supplementary Fig. 2c**). SOCE deficient cells showed a severe defect in mRNA expression of uncoupler protein 2 (UCP2) (**Fig.2e**), a proton transporter in the inner mitochondrial membrane, and this defect was confirmed at the protein level (**Fig.2f**). Opening of UCP2, which is controlled by fatty acids and other factors, results in the reduction of the mitochondrial membrane potential (MMP) and ROS production and thus prevents mitochondrial damage[26,27]. Consistent with reduced UCP2 expression and higher numbers of damaged mitochondria in fibroblasts of SOCE-deficient patients we observed increased co-localization of mitochondria and lysosomes (**Fig. 2g**), indicative of a higher frequency of mitophagy events. Similar results were found by using the pH sensitive MitoKeima reporter[28], which detects mitochondria undergoing degradation in acidic compartments (**Supplementary Fig. 3**). Similarly to patient fibroblasts, we observed higher numbers of damaged mitochondria in the skeletal muscle and hearts of *Orai1*^{R93W} knock-in mice by TEM (**Supplementary Fig. 1a-c**)[22]. Also consistent with reduced UCP2 expression in SOCE deficient cells, we observed significantly higher basal MMP levels in patient compared to control cells using tetramethylrhodamine (TMRM) as a MMP-sensitive dye (**Fig.2i**).

To assess the function of the ETC in mitochondria of SOCE-deficient cells, we measured the hyperpolarization rate of the MMP after treatment of cells with oligomycin, which inhibits the ATP synthase and thus traps protons in the intermembrane space. We found a reduced proton pumping rate by the ETC in patient compared to control cells (**Fig.2j**). We next analyzed the electron transport rate of the ETC by measuring the formation of O_2^- using the superoxide-sensitive, mitochondria-targeted dye MitoSOX. Inhibition of the ATP-synthase with oligomycin forces electrons produced by the ETC to form O_2^- with a rate that is proportional to the rate of electron transport. Whereas basal mitochondrial superoxide levels were slightly, but not statistically significantly increased in SOCE-deficient cells (**Fig.2l**), the rate of superoxide production after ATP synthase inhibition was significantly reduced in the absence of SOCE indicative of lower electron-transport rate by the ETC (**Fig.2m**). In addition, we directly analyzed the O_2 consumption rate in fibroblasts from 2 patients and one healthy donor as a measure of cellular respiration. Whereas basal respiration rates were normal or moderately reduced in patient fibroblasts, we observed a significant decrease in the maximal respiration rates in cells from both SOCE-deficient

patients compared to controls (**Supplementary Fig. 2a**). Reduced ETC function was not specific to fibroblasts because a functional analysis of individual ETC complexes in skeletal muscle biopsies of ORAI1-deficient patients revealed decreased activity of complexes I and IV in two out of three patients (**Supplementary Table 1**).

Ca^{2+} is a co-factor of three enzymes in the TCA cycle[2-4], and impaired ETC function in SOCE-deficient cells might therefore be due to decreased levels of reduced co-enzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). However, SOCE-deficient cells showed significantly increased absolute levels of NADH (**Fig. 2n,o**). Furthermore, they had no defect in replenishing the NADH pool after its depletion with FCCP and subsequent blockade of the electron acceptor complexes I and III with antimycin A and rotenone (**Fig.2n,p**). These findings indicate that the TCA cycle is not dependent on SOCE, at least under the experimental conditions used here, and does not account for impaired ETC function in SOCE-deficient cells. An important function of the mitochondria is to break down fatty acids into acetyl-CoA by β-oxidation. Several enzymes are critical for this process including acyl-CoA dehydrogenase very long-chain (ACADVL), an enzyme that catalyzes the first step of β-oxidation, and the long-chain fatty acid transporter carnitine palmitoyl–transferase 1B (CPT1B) that facilitates the transport of fatty acids into the mitochondria[29]. Expression of both genes was reduced in patient fibroblasts when cultured in high-glucose medium, and this difference became more apparent when cells were cultured in oleic acid-containing medium (**Fig.2q,r**). Taken together, these data show that SOCE controls expression of several key mitochondrial enzymes including uncoupler proteins, ETC components and β-oxidation enzymes and thereby regulates mitochondrial function.

Cells can store excess energy as triacylglycerol (TAG) in LDs, that can be mobilized by cytosolic neutral lipases, which hydrolyze TAG into free fatty acids (FFA) and glycerol for mitochondrial utilization[30]. Defective lipolysis leads to accumulation of LDs in the heart muscle and other tissues[31]. We tested the hypothesis that in addition to the alterations seen in mitochondrial function, changes in the breakdown of TAG by lipolysis could account for accumulation of LDs in SOCE-deficient cells. Fibroblasts from patients with loss-of-function mutations in *ORAI1*or *STIM1* were cultured in high glucose or oleic acid-containing media and lipolysis was induced by starvation of cells in the presence or absence of the adrenergic stimulus isoproteranol. Patient fibroblasts showed impaired lipolysis measured by free-glycerol release when cultured in high-glucose medium and were defective in the upregulation of lipolysis after challenge with oleic acid (**Fig.3a**). This defect could not be rescued by the β-adrenergic receptor agonist isoproteranol (**Fig.3a**). Similar observations were made in mouse NIH3T3-L1 cells that express a dominant negative mutant form of ORAI1 (ORAI1-E106Q) that abolishes SOCE (**Fig.3b**). SOCE-deficient NIH3T3-L1 cells showed significantly reduced lipolysis in high-glucose medium and showed impaired upregulation of lipolysis after challenge with oleic acid (**Fig.3c**). The lipolysis defect was not dependent on the saturation of the fatty acid nutrient, as SOCE-deficient cells cultured with palmitic instead of oleic acid also failed to upregulate lipolysis (**Fig.3d**). We conclude that SOCE is required for basal lipolysis and for the upregulation of lipolysis after fatty acid challenge.

In addition to conventional lipolysis, LDs can also be targeted for lysosomal hydrolysis through a selective form of autophagy called lipophagy[32]. LDs delivered to lysosomes for degradation by acidic lipases result in the production of FFA that can be utilized for β-oxidation by mitochondria[32]. Because lipophagy is an alternative pathway to conventional lipolysis in the regulation of intracellular lipid stores, we investigated if SOCE deficiency in patient fibroblasts affects the status of the autophagy/lysosomal

system. Immunoblots for the autophagosome marker microtubule-associated protein light chain 3 (LC3) in cells maintained in the presence or absence of lysosomal protease inhibitors revealed consistently higher steady-state levels of LC3-II and increased autophagic flux (measured as the amount of LC3-II degraded in lysosomes) in patient cells, suggesting higher rates of autophagy in the absence of SOCE (**Fig. 4a,b**). We confirmed this finding using a tandem fluorescent LC3 construct that allows for measuring autophagosome content (as $mCherry^+$ and GFP^+ fluorescent puncta) and autophagosome maturation into autolysosomes (as $mCherry^+$ only puncta). Consistent with the immunoblot data, SOCE-deficient cells had significantly higher content of autophagosomes and an even more pronounced increase in autolysosomes (**Fig. 4c,d**). Levels of other autophagy-related proteins (Atg5/12, p62, LAMP1) and of the transcription factor EB (TFEB) that controls the autophagic and lysosomal transcriptional programs, were also significantly higher in SOCE-deficient patient cells (**Supplementary Fig. 4**). We found similar upregulation of autophagy in the absence of SOCE in the skeletal muscle of *Orai1*^{R93W} knock-in mice. Morphometric analysis of TEM images revealed a significant increase in the number of autophagic vacuoles in knock-in compared to WT mice (**Supplementary Fig. 5a,b)**.

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As in human fibroblasts, the higher content of autophagic compartments in muscles of knock-in mice was a result of higher number of autolysosomes, further supporting an increase in autophagic flux in these animals (**SupplementaryFig. 5c**). Despite marked upregulation of autophagy in SOCE-deficient cells, we found that it is not targeting protein cargo, as proteolysis of long-lived proteins was reduced in the absence of SOCE (**Fig. 4e**) and this reduction was preferentially due to decreased 3-Methyladenine(3-MA)-sensitive proteolysis (macroautophagy)(**Fig. 4f**). We next investigated lipophagy in SOCE-deficient cells upon oleic acid challenge by measuring the co-localization of LDs with LC3. Both the percentage of LDs decorated with LC3 (**Fig. 4g,h**) and the percentage of autophagic vacuoles containing lipid as cargo (**Fig. 4g,i**) were significantly higher in SOCE-deficient cells, suggesting that the increased number of autophagosomes in patient cells is due to increased lipophagy. Analysis of the LDs that accumulate in the skeletal muscle from *Orai1*^{R93W} knock-in mice also revealed that a significantly higher percentage of LDs in these animals had morphological features previously attributed to active lipophagy (**Supplementary Fig. 5d-f)**. To assess the functional importance of increased lipophagy in SOCE-deficient cells, we ablated expression of autophagy-related protein 7 (ATG7), which is essential for autophagosome formation, using specific short-hairpin RNA. Deletion of ATG7 (**Fig. 4j**) rendered SOCE-deficient cells more susceptible to oleic acid and palmitic acid induced lipotoxicity, whereas it had no effect on the viability of lipid-challenged WT cells or the sensitivity of patient cells to high glucose levels (**Fig. 4k**). Taken together, these results suggest that the increase in lipophagy in SOCE-deficient cells was elicited as a protective mechanism against lipotoxicity.

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Mammals adapt to prolonged fasting by activating lipolysis in their adipose tissues, thereby releasing large amounts of fatty acids into the circulation[33]. To adjust to the abundance of fatty acids the liver and other organs have to reprogram their metabolism by transcribing genes involved in fatty acid oxidation. PPARα[34,35] and its co-regulator PGC-1α[36,37]were identified as the transcriptional regulators of the fasting response in the liver and the heart; however, the upstream signaling events leading to metabolic reprogramming are still incompletely understood. We hypothesized that SOCE may be involved in sensing extracellular fatty acids, and subsequently in metabolic reprogramming, potentially explaining the defect in lipid metabolism in the absence of SOCE. HD fibroblasts responded to oleic acid challenge with a sustained elevation in intracellular Ca^{2+} levels that was almost completely absent in fibroblasts from

patients with loss-of-function mutations in *ORAI1* or *STIM1*, indicating that oleic acid induces SOCE (**Fig. 5a**). The relevance of this fatty acid-induced SOCE was tested first in vitro by measuring PGC-1α and PPARα expression in fibroblasts before and after oleic acid challenge. We observed a significant reduction in basal levels of PGC-1α and PPARα in SOCE-deficient fibroblasts, and an impaired induction of expression upon oleic acid challenge (**Fig. 5b**). Similar results were obtained when we analyzed SOCE-deficient NIH3T3-L1 cells expressing a dominant negative ORAI1-E106Q channel (**Fig. 5c**), suggesting a conserved role of SOCE in the regulation of PGC-1α and PPARα expression. Interestingly, low PPARα expression may also contribute to the induction of autophagy observed in SOCE-deficient cells as PPAR agonists suppressed the increased autophagy flux in SOCE-deficient patient fibroblasts (**Supplementary Fig. 6a**) and in cells expressing ORAI1-E106Q (**Supplementary Fig. 6b**) to levels comparable to their respective controls cells.

SOCE may be sufficient on its own to induce PGC-1α expression because induction of SOCE by passive depletion of the ER Ca^{2+}stores with the sarco/endoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin resulted in levels of PGC-1αexpression that were comparable to those observed after culturing cells in oleic acid-containing medium (**Fig. 5d**). Induction of PGC-1α expression by thapsigargin was partially dependent on the activity of calcineurin and adenylate cyclases, because it was inhibited by FK506 and SQ22,536, respectively, whereas CAMK inhibition with KN93 had no inhibitory effect on PGC-1α expression (**Fig. 5d**). We observed a similar dependence of PGC-1α and PPARα expression on SOCE in vivo (**Fig. 5e,f**). After we abolished SOCE in *Stim1$^{fl/fl}$Stim2$^{fl/fl}$ UBC-ER^{T2}-Cre* mice by tamoxifen injection, we found significantly reduced expression of PGC-1α and PPARα in the heart of mice 5 days after the first injection (**Fig. 5e**). Fasting results in increased fatty acid levels in the serum and induces fatty acid-mediated reprogramming of tissue metabolism to adapt to nutrient changes[38]. To test if adaptation to lipid metabolism upon fasting is associated with increased expression of CRAC channel genes, we measured expression of STIM1 and ORAI1 in mice fed a normal diet and mice fasted for 24 hours. We observed increased expression of PGC-1α and PPARα in the liver and heart of fasted WT animals (**Fig. 5g,h**). Fasted animals also significantly upregulated expression of STIM1, but not ORAI1, in their heart and liver (**Fig. 5g,h**). Taken together, these findings show that fatty acids induce SOCE, which is required for the expression of PGC-1α and PPARα, transcriptional regulators that control metabolic reprogramming in circumstances when fatty acids are in abundance. The expression of STIM1 is upregulated under fasting conditions, presumably in an effort to adapt to enhanced lipid metabolism.

Discussion

The increasing incidence of metabolic diseases including obesity, diabetes and cardiovascular diseases presents an unresolved threat to human health. Identifying the molecular regulators of lipid metabolism may provide better insights into the pathogenesis of these diseases. Ca^{2+} has been identified as a regulator at the intersections of several metabolic pathways[1]. However, the Ca^{2+} channels that mediate Ca^{2+} influx and control Ca^{2+} dependent metabolic pathways remain largely unknown.

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Here we identify CRAC channels and the SOCE they mediate as regulators of lipid metabolism. We find that ORAI1- and STIM1/2-deficient mice with abolished SOCE present with abnormal accumulation of LDs in the heart, liver and skeletal muscle, a phenotype that in many aspects phenocopies PGC-1α[39] and PPARα[34,35] knock-out mice. We show that the role of SOCE in lipid metabolism is at least partially cell intrinsic, as fibroblasts from human patients with loss-of-function mutations in *STIM1* or *ORAI1* show similar deregulation in lipid balance. Our findings are consistent with a recent study that identified CRAC channel genes as regulators of adiposity in Drosophila[40].

Intact mitochondrial function is crucial to prevent triglyceride accumulation in tissues such as skeletal muscle and liver[23]. Intracellular Ca^{2+} has been shown to control the function of several TCA-cycle enzymes in mitochondria[2-4]. However, we find that SOCE is dispensable for the TCA-cycle function, at least under the conditions used here, and instead regulates the function of the electron transport chain. SOCE-deficient patients present with reduced activity of ETC complexes I and IV and decreased expression of key components in both complexes, *NDUFA1* and *COX4I1*. Despite reduced ETC function, the mitochondrial membrane potential is increased in the absence of SOCE, most likely because of strongly decreased expression of UCP2, which allows protons to reenter the mitochondrial matrix bypassing the ATP synthase. Mitochondria in SOCE-deficient cells were also defective in expression of *CPT1B* and *ACADVL*, genes involved in beta-oxidation of fatty acids. Expression of all these genes is controlled by the transcriptional regulators PGC-1α and PPARα. The levels of both factors were reduced in the absence of SOCE. It is noteworthy that despite impaired expression of PGC-1α, and its reported role in mitochondrial biogenesis[41], we found normal numbers of mitochondria in SOCE deficient cells, possibly due to a compensatory upregulation of PGC-1β (**Supplementary Fig. 7).**

While SOCE controls several aspects of mitochondrial function, its role in lipid metabolism in not limited to the mitochondria. We identify CRAC channels as essential regulators of basal lipolysis and fatty acid challenge-induced upregulation of lipolysis. Fatty acid transporters, such as CD36, activate phospholipases[42] that convert PIP_2 into IP_3, potentially resulting in the emptying of ER Ca^{2+} stores, activation of STIM1 and SOCE. Indeed, STIM1 has recently been described to be indispensable for the fatty acid-induced Ca^{2+} response in mouse taste-bud cells[43]. Here we show that responsiveness of CRAC channels to fatty acids is preserved in human cells, and is crucial for induction of lipolysis and PGC-1α and PPARα expression both in vitro and in vivo. In the absence of SOCE, fatty acids cannot induce the expression of PGC-1$\alpha$and PPARα and as consequence cells fail to transcribe genes important for lipid metabolism, which in turn leads to accumulation of lipid droplets and induction of lipophagy in an effort to protect cells from lipotoxicity. Our results describe a novel regulatory pathway of lipid metabolism that connects extracellular fatty acid-induced SOCE to metabolic reprogramming (**Supplementary Fig.8**). The tissue-specific role of SOCE in lipid metabolism and the nutrients and growth factor receptors activating SOCE in specific tissues remain to be explored.

Materials and methods

Mice. *Orai1*^{R93W}knock-in mice[22] and *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ mice[44] have been described previously. *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ mice were crossed with B6.Cg-Tg(UBC-Cre/ERT2)1Ejb/J (*UBC-ER*^{T2}*-Cre)* mice (The Jackson Laboratory, strain 008085). Mice were fed regular chow and allowed *ad libitum* access to food and water except when stated otherwise. For fasting, mice were deprived from access to food for a period of 24 hours. For inducible deletion of the loxP-flanked *Stim* alleles, tamoxifen (Sigma Aldrich) was freshly dissolved in corn oil. 1 mg tamoxifen/20 g body weight was administered to *Stim1*$^{fl/fl}$ *Stim2*$^{fl/fl}$ *UBC-ER*^{T2}*-Cre*$^{+}$mice and their *Cre*$^{-}$littermates by i.p. injection daily for 5 consecutive days. All animal experiments were conducted in accordance with protocols approved by the IACUC of New York University School of Medicine.

Cells and cell culture. Platinum E (Plat-E) cells for retroviral packaging, HEK293 FT cells for lentiviral packaging and NIH3T3-L1 were cultured in DMEM (Mediatech) at 37°C, 10% CO_2. SOCE-deficient fibroblasts were used from three unrelated patients: P1 was described previously and is homozygous for a R429C missense mutation in STIM1[19,45]. P2 was described previously and is homozygous for a R91W missense mutation in ORAI1[12]. P3 has not been previously reported and is homozygous for a G98R missense mutation in ORAI1 that abolishes ORAI1 protein expression and SOCE. Fibroblasts from patients and from healthy donors were immortalized by retroviral transduction with human telomerase reverse transcriptase (hTERT) as previously described[12] and cultured in RPMI 1640 in (Mediatech) at 37°C, 10% CO_2. DMEM and RPMI 1640 were supplemented with 10% (vol/vol) FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all from Mediatech). For studies of mitochondrial respiration, control fibroblasts from healthy donors were obtained from the Biobank of the Medical Research Council (MRC), Centre for Neuromuscular Diseases, Newcastle and were immortalised as described (Lochmüller et al., 1999). Fibroblasts were grown in high glucose DMEM (Sigma, Poole, UK) supplemented with 10% (v/v) fetal bovine serum and 200 µM Uridine (Sigma).

Plasmids and Reagents. Human dsRed-LC3 plasmid[46], mtKeima plasmid[28], GFP-dgn/GFP-degFS[47], ORAI1-E106Q[48] plasmids were described before. Cells were transfected with 1µg DNA plasmid (or 0.5µg of each plasmid in case of co-transfection) using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. Sources of chemicals were as previously described[32,49,50]. Neutral lipids were stained with Bodipy 493/503 (D-3922) (Molecular Probes) as described[32]. Where indicated, cells were incubated with LysoTracker® Red DND-99 (L-7528), MitoTracker® Green FM (M-7514) (Molecular Probes), MitoSOX (Molecular Probes) according to the manufacturers' instructions. Oleic acid (Sigma Aldrich) was conjugated to bovine serum albumin (BSA) as described[32]. Palmitic acid (Sigma Aldrich) was dissolved in chloroform and conjugated to BSA after evaporation of chloroform as described for oleic acid. Cells were treated with 0.125mM oleate or palmitate for 24h unless otherwise stated. Oligomycin, carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), KN93, SQ22,536 were purchased from Sigma Aldrich, FK506 was purchased from Tocris.

Histology and Oil Red O staining. For Oil Red O staining, tissues were flash frozen in OCT and stored at -80°C prior to sectioning. 5 µm cryosections were dried, fixed briefly in 10% neutral buffered formalin, washed with water three times and then 60% isopropanol before staining with 0.3 % Oil Red O in 60%

isopropanol for 15 minutes. Sections were rinsed with 60% isopropanol and counterstained with Mayer's hematoxylin and mounted with glycerin jelly mounting medium.

Tricarboxylic acid (TCA) cycle analysis. TCA cycle function was measured as previously described[51]. Briefly, steady-state levels and dynamic changes of NADH levels were analyzed by measuring NADH autofluorescence using time-lapse fluorescence microscopy. Fibroblasts from healthy donors as well as ORAI1- and STIM1-deficient patients were seeded in 8-well cover glass chambers (Thermo Scientific) at a density of 20,000 cells per well and incubated at 37°C and 5% CO_2 overnight. 2 hours prior to experiments, cell culture medium was replaced with glucose-free RPMI 1640 medium supplemented with 5 mM sodium pyruvate, 1% HEPES, and 1% Penicillin / 1% Streptomycin. Immediately before NADH measurements, cells were washed three times with modified glucose-free Ringer's solution containing 5 mM sodium pyruvate and 1.2 mM $CaCl_2$. Autofluorescence of cells was recorded using an Olympus IX-71epifluorescence microscope and a 40x, 1.35 N/A oil objective. NADH autofluorescence was measured at an emission wavelength of 461 nm after excitation at 358 nm. FCCP (1µM)and a mixture of antimycin A (1µM) and rotenone (100nM) was pipetted into the chamber manually at the indicated time points. Images were analyzed using NIH ImageJ software.

Electron conductance rate of the electron transport chain(ETC). Cells were stained with the mitochondrial superoxide sensitive dye MitoSOX (Invitrogen) according to the manufacturer's instructions. Oligomycin (1µM) was used to inhibit the ATP-synthase. At indicated time points, cells were brought in suspension using 0.05% Trypsin plus 0.53mM EDTA, fixed in 4% paraformaldehyde, and MitoSOX fluorescence was measured using flow-cytometry. The electron transport rate was calculated as the slope of MitoSOX fluorescence as function of time after oligomycin treatment based on the assumption that the electrons transported by the ETC are forced to form superoxides at a rate that is theoretically proportional to the electron conductance rate of the ETC.

Mitochondrial membrane potential (MMP). For MMP measurements cells were grown on UV-sterilized 15 mm diameter glass coverslips. Tetramethylrhodamine (TMRM)(Biovision) was used in a non-quenching mode (20 nM) as described[51]. Experiments were performed at room temperature (22–25 °C) in a RC-20 perfusion chamber (Warner Instruments). Cells were analyzed by time-lapse imaging on an IX81 epifluorescence microscope (Olympus). A laser line (543 nm; Melles Griot) was used to excite TMRM, and fluorescent images were acquired in 5-s intervals. Image analysis was conducted using Slidebook v4.2 (Olympus) and ImageJ (NIH) imaging software. For scaling the TMRM fluorescence to reference MMP values, TMRM fluorescence obtained after hyperpolarization of the mitochondria with the ATP synthase inhibitor oligomycin (1µM) was set to 1, and TMRM fluorescence obtained after depolarization of the mitochondria with the proton ionophore FCCP (1µM) was set to 0.The proton pumping rate was calculated as the slope of hyperpolarization after addition of the ATP synthase inhibitor oligomycin, in the linear phase before reaching plateau.

Oxygen consumption and respiratory chain enzyme function analysis. Measurement of intact cellular respiration was performed using the Seahorse XF24 analyzer (Seahorse Bioscience). Fibroblasts were plated at a density of 18,000 cells / well on XF24 tissue culture plate. Prior to the respiration assay, cells were rinsed and cultured in DMEM running medium (8.3 g/L DMEM (Sigma), 200 mM GlutaMax-1 (Invitrogen), 100 mM sodium pyruvate (Sigma), 25 mM D-glucose (Sigma), 63.3 mM NaCl (Sigma), and phenol red (Sigma), adjust pH to 7.4 with NaOH) according to manufacturer's protocol. Cells were

incubated at 37°C in a CO_2-free incubator for one hour prior to measurements. Oxygen consumption was measured under basal conditions and in the presence of the mitochondrial uncoupler FCCP (3 μM, Sigma) to assess maximal oxidative capacity. To normalize respiration rates to cell numbers, cells were fixed with 4% paraformaldehyde and counted after nuclear staining with Hoechst 33258 (1g/ml, Invitrogen). The respiratory chain enzyme activity in muscle biopsies from patients with loss-of-function mutations in ORAI1 (Supplementary Table 1) was measured as previously described (Gempel et al., 2007; Kirby et al., 2007).

Lipolysis. Lipolysis was measured indirectly by quantifying free-glycerol release into the extracellular-media. Cells were plated in24-well plates and cultured overnight in RPMI 1640 medium (fibroblasts) or DMEM medium (NIH3T3-L1 cells) containing 10 mM glucose and supplemented with 10% fetal bovine serum (FBS) (high glucose media). Alternatively, cells were cultured in RPMI 1640 (fibroblasts) or DMEM (NIH3T3-L1 cells) containing 0.5 mM oleic acid-BSA (alternatively 0.5 mM palmitic acid-BSA), 1% FBS and 1 mM glucose. The next morning, medium was replaced with RPMI 1640 containing 1mM glucose with or without 1μM isoproterenol as indicated. Fibroblasts were cultured for an additional 24 hours and NIH3T3-L1 cells for an additional 6 hours. Supernatant was removed and glycerol content was measured with a colorimetric assay using the free glycerol reagent (F6428, Sigma Aldrich). Glycerol levels were normalized to cell numbers for all experiments.

Lipotoxicity. Cells were plated in 24 well plates and cultured for 24 hours in RPMI1640 mediumcontaining10 mM D-glucose (glucose media), or 1 mM oleic acid-BSA plus 0.5 mM D-glucose (oleic acid medium) or 0.5 mM palmitic acid-BSA plus 1 mM D-glucose (palmitic acid medium). 24 hours later adherent cells were brought in suspension using 0.05% Trypsin + 0.53mM EDTA and collected together with floating cells. Cells were stained with 1 μg/ml propidiumiodide (PI) and analyzed by flow cytometry. Cell death was calculated as the percentage of PI^+ cells.

Quantitative real-time PCR. Total RNA from cells and homogenized tissue was isolated using TrIzol (Invitrogen), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed using the TaqMan probe method with premade primers (Applied Biosystems) in case of mouse β-actin, mouse PGC1α and mouse PPARα, for all other targets PCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo). The specificity of the PCR was verified by melt curve analysis. The relative abundance of transcripts was normalized to the expression of housekeeping genes using the 2-ΔΔCT method. Relative expression values presented were normalized to the average of untreated WT mouse or healthy donor human controls. The specific primers and probes used are listed in Supplementary Table 2 .

Mitochondrial DNA copy number. Total DNA from cells was isolated using the FlexiGene DNA kit (Qiagen). Oligonucleotide probes were designed against 3 different regions of mtDNA, and 2 regions of genomic DNA. Specific primers used are listed in Supplementary Table 2. Quantitative RT-PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo). mtDNA copy number was calculated relative to genomic DNA as previously described[52].

Intracellular Ca^{2+} measurements were conducted in 96-well format using a FlexStation 3 multi-mode microplate reader (Molecular Devices) or by single cell Ca^{2+} imaging using an IX81epifluorescence microscope (Olympus). For FlexStation measurements, NIH3T3-L1 cells were grown in translucent 96-well plates (BD Falcon), stained with 1μM Fura-2-AM and washed twice before measurements. Cells

were kept in Ca^{2+}free Ringer solution (in mM: 155 NaCl, 4.5 KCl, 3 $MgCl_2$, 10 D-glucose, 5 Na-Hepes pH 7.4)at the beginning of the measurement. 1 μM thapsigargin (Calbiochem) was used to induce passive store depletion. Ringer solution containing 2 mM Ca^{2+} was added to cells to obtain a final extracellular Ca^{2+}concentration of 1 mM and to induce SOCE. Fura-2 emission was measured at 510 nm after excitation at 340nm (F340) and 380nm (F380). The intracellular Ca^{2+} concentration is proportional to the fluorescence emission ratio F340/F380.For single cell Ca^{2+} imaging, fibroblasts were grown on UV-sterilized 15 mm glass coverslips, loaded with 1 μM Fura-2-AM, mounted in a perfusion chamber and analyzed as described above. All experiments were performed at room temperature (22–25 °C). Where indicated, Ringer solution was replaced with 1 mM Oleic-BSA and 1 mM Glucose containing RPMI media to measure Oleic-BSA induced changes in the intracellular Ca^{2+} concentration. Image analysis of > 20 cells per experiment was conducted using Slidebook imaging software v4.2. (Olympus).

Transmission electron microscopy. Sacrificed mice were fixed by cardiac perfusion with 4% paraformaldehyde/ 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3. After dissection, fixation was allowed to continue at room temperature for 90 min or 4˚C overnight. Cardiac and skeletal muscle tissue was washed with 100 mM Tris (pH 7.2) and 160 mM sucrose for 30 min followed by washes in iso-osmotic phosphate buffer (150 mM sodium chloride, 5 mM potassium chloride, 10 mM sodium phosphate, pH 7.3) for 30 min twice. After treatment with 1% osmium tetroxide in 140 mM sodium phosphate (pH 7.3) for 1 h, muscle tissue was washed (twice for 1 h) in water. The muscle was stained en bloc with saturated uranyl acetate for 1 h, dehydrated in ethanol, and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA). Semi-thin sections were cut at 1 mm and stained with 1% toluidine blue to evaluate the quality of preservation. Ultrathin sections (60 nm) were cut and stained with uranyl acetate and lead citrate by standard methods. Stained grids were examined using a Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan Erlangshen ES1000W digital camera (Model 785, 4 k 3 2.7 k; Gatan, Pleasanton, CA). Morphometric analysis of mitochondria, lipid droplets and autophagic compartments were done by double-blinded independent observers in at least 8 different micrographs per condition. Organelle areas were calculated by NIH ImageJ software after tracing the membrane profiles using the measure function. Classification of autophagic vacuoles as autophagosomes or autolysosomes was done using standard criteria [53]using a single-category allocation for each vesicle.

Autophagy flux analysis. Measurements of intracellular protein degradation were performed by metabolic labeling (with [^{3}H] leucine (2 μCi/ml) (NEN PerkinElmer Life Sciences) for 48hrs at 37°C and pulse-chase experiments as described before[54]. After labeling, cells were extensively washed and maintained in medium with an excess of unlabeled leucine (*S6*). Aliquots of the medium taken at different times were precipitated in trichloroacetic acid and proteolysis measured as the amount of acid-precipitable radioactivity transformed in acid-soluble radioactivity at each time. Macroautophagy-dependent degradation was inhibited using 3-methyladenine (10mM) (Sigma) and lysosomal-dependent degradation was inhibited using the mixture ammonium chloride (20mM) (American Bioanalytical) and Leupeptin (100μM) (Fisher Bioreagents) (N/L).

Autophagy activity was estimated by: (i) immunoblot for LC3-II in cells untreated or treated with the inhibitor mixture N/L for the indicated times. The level of LC3 lipidation was quantified as the ratio of the measured LC3-II to Actin levels. LC3 flux was quantified as the relative ratio of LC3-II/actin values between samples with or without the lysosomal proteolysis inhibitors. (ii) direct fluorescence in cells

transduced with lentivirus carrying the mCherry-GFP-LC3 tandem construct. Cells were plated in coverslip or glass-bottom 96-well plates and fluorescence was read in both channels. Puncta positive for both fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the conversion of autophagosomes to autolysosomes (red only puncta). Where indicated cells were plated in 96 well- glass bottom plates and imaged with a high-content microscope (Operetta system, Perkin Elmer).

Antibodies. The following commercially available antibodies were used: LAMP1 and LAMP2 (both Developmental Studies Hybridoma Bank); ATG5 (Novus Biologicals); LC3 (Cell Signaling); Actin (Abcam); ATG7 (Cell Signaling); UCP2 (Santa Cruz); NDUFB8, Core2, COX1, COX2, COX4, SDHA α subunit and porin (all from Mitosciences), cytochrome c (BD Biosciences), β□actin (Sigma Life Science).

Immunofluorescence microscopy. Cells grown on coverslips were fixed with 4% paraformaldehyde (or pre-chilled methanol in case of staining for Hsc70, in order to highlight the membrane-associated protein) for 30min, permeabilized and blocked with 1% BSA, 0.01% Triton X-100 in phosphate buffer saline (PBS). Incubation with primary and secondary antibody conjugated to Alexa Fluor 488 or Cy5 (Invitrogen) in 0.1% BSA in PBS was performed at room temperature for 1h each. For LD staining, cells were incubated with BODIPY 493/503 (Invitrogen, 20mg/ml) or 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich, 5mM; pseudo-colored to red or green) for 20min at 37°C prior to fixation. For mitochondria staining, cells were incubated with MitoTracker (50mM) and MitoTracker CMXRos (50nM) (Invitrogen) for 20min 37°C prior to fixation. Coverslips were mounted in DAPI-Fluoromount-G (Southern Biotechnology) or Draq5 (Invitrogen; pseudo-colored to blue) to stain the nucleus. All images were acquired with Axiovert 200 fluorescence microscope (Carl Zeiss Microscopy) with ×63 objective and 1.4 numerical aperture, mounted with an ApoTome.2 slider, and prepared using Adobe Photoshop CS3 (Adobe Systems) and ImageJ (NIH). For high-content microscopy, cells were plated in 96-well plates, fixed and stained using the same procedures but images were captured with a high-content microscope (Operetta system, Perkin Elmer) and quantification was performed with the manufacturer's software in a minimum of 800 cells (approx. 9 fields). In all cases, focal plane thickness was set at 0.17 mm (average cell thickness was 1mm) and sections with maximal nucleus diameter were selected for quantification. Values are presented as number of puncta per cell section that in our acquisition conditions represents 10-20% of the total puncta per cell. The number of particles/puncta per cell was quantified using 'analyze particles' function of ImageJ after thresholding in non-saturated images. The percentage of co-localization was determined by 'JACoP' plugin in ImageJ after thresholding of individual frames[55]. Where indicated, the co-localized pixels were highlighted using 'co-localization' plugin of ImageJ.

RNAi interference. The lentiviral shRNA constructtargetingATG7 was generated as described before[56]. Briefly, the hairpin (sense-loop-antisense) against the ATG7 sequence 5'-GGTGTTTAACTCTTCACAT-3' was inserted into the multicloning region of the pSUPER vector (Ambion, Austin, TX) as described[57]. The H1-promoter along with the hairpin sequence were removed from the pSUPER vector by digestion with the restriction enzymes SmaI and Xho I and ligated between the EcoRV and XhoI sites of the pCCL.PPT.hPGK.GFP.Wpre lentiviral transfer construct. For virus production a HEK293 FT cell line was used that had been previously stably transduced with the lentiviral *gag* and *pol* genes. HEK293 FT cells were co-transfected with the lentiviral shRNA construct and a VSV-G packaging construct containing the lentiviral *env* gene. Viral supernatants were collected on days 2 and 3 after transfection. Viral

Commented [F 1]: Mate: what is the source of the cell line and who made it? Where is the rev gene coming from?

supernatants were mixed with RPMI 1640 plus 10% FBS at a 1:4 ratio. 8 μg/ml polybrene was added to the medium. Knock-down efficiency of ATG7 was tested by Western blotting.

Western blotting. Total cell lysates were prepared in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% NaDoc, 0.1% SDS, 50mM Tris pH 8) containing protease and phosphatase inhibitors. Protein concentrations of lysates were measured using BSA as a standard[58]. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, blocked with low-fat milk and incubated with primary antibody overnight. The proteins were visualized with peroxidase-conjugated secondary antibodies and chemiluminescent reagent (PerkinElmer) using a LAS-3000 Imaging System (Fujifilm). Densitometric quantification was performed on unsaturated images using ImageJ. Mitochondrial proteins from immortalized fibroblasts were isolated by differential centrifugation, solubilized by dodecylmaltoside (DDM, Sigma) at 2 g/g protein and incubated for 20 min on ice. The supernatant was collected after 20 min centrifugation at 25.000xg, and to resolve individual complexes and smaller supercomplexes, 25 μg of DDM treated mitochondrial membrane proteins were run on 4.5% -16% gels (Wittig et al., 2006). After electrophoresis, the complexes were transferred to PVDF membranes and sequentially probed with antibodies.

Statistical Analyses. All numerical results are reported as mean ± standard error of the mean (SEM). The statistical significance of the difference between experimental groups was determined by two-tailed unpaired Student's *t*-test. Where multiple comparisons were performed, we used normalization to control values. Differences were considered significant for $P<0.05$ (noted in the figures as *). The exact sample size (n) is indicated in each figure and it corresponds to individual experiments unless otherwise stated. All experiments were done at least 3 times and in duplicate or triplicate to account for technical variability. For the morphometric analysis quantification of annotated micrographs were independently reviewed by two observers and the average of their scoring was used for each micrograph. For immunofluorescence and direct fluorescence quantifications of co-localization, numbers of puncta per cell and the ratio of red to green fluorophore were performed blinded.

Figure legends

Figure 1. SOCE regulates lipid balance in cells and tissues. (**a-c**) Lipid droplet accumulation in organs of SOCE-deficient mice. EM images of heart (**a**) and Oil Red O stain of skeletal muscle sections (**b**) from wild-type and *Orai1*^{R93W}knock-in mice 5 days postpartum.(**c**) Oil Red O stain of heart, liver and soleus sections (10x) from 16-week-old *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$*UBC-ER*^{T2}*-Cre*mice and Cre-negative littermates 5 days after the first dose of tamoxifen injection. (**d**) EM image (20,000x) of a skeletal muscle biopsy obtained from a patient with ORAI1-G98R loss-of-function mutation shows lipid droplet deposition (arrow). (**e**) SOCE in fibroblasts from healthy donors (HD, blue) and patients with loss of function mutations in *STIM1* or *ORAI1* (P, red) measured by single-cell Ca^{2+} imaging. ER Ca^{2+} stores were passively depleted with thapsigargin (TG) and SOCE was measured after perfusion with Ringer's solution containing 2 mM Ca^{2+}. Traces represent the averages obtained from 3 HDs and 3 patients. At least 20 cells per donor were analyzed. (**f, g**) Neutral lipid staining of fibroblasts from HD and P cultured in high glucose medium. Cells were stained with Bodipy 493/503, and fluorescence was measured by flow cytometry. Representative histograms (**f**) and average mean fluorescent intensities (MFI)(**g**) of Bodipy 493/503 after subtraction of autofluorescence (ΔMFI) in 3 HD and 3 P fibroblasts (left) and values averaged for all HD and P fibroblasts (right). Values in (**g**) are normalized to the mean value obtained in HD fibroblasts. (**h**) HD and P fibroblasts were challenged with oleic acid (0.125 mM, 24 hours) and stained with Bodipy 493/503 (green) to identify neutral lipid stores. Dashed boxes indicate regions shown at higher magnification in the right panels. Nuclei were counterstained with DAPI. (**i**) Average numbers of LDs per cell in HD and P fibroblasts (left), percentage of cellular area occupied by LD (middle) and average LD area per cell (right). All values represented by bar graphs are mean ±SEM. Statistical analysis was performed using Student's t-test. * $p<0.05$.

Figure 2. SOCE is required for mitochondrial function. (**a, b**)The mitochondrial volume was measured in fibroblasts from healthy donors (HD, blue) and patients with loss-of-function mutations in *STIM1* or *ORAI1* (P, red) by staining with Mitoview Green and analysis by flowcytometry. Representative histograms (**a**) and average mean fluorescent intensity (MFI) of MitoView Green after subtraction of autofluorescence (ΔMFI) in HD and P fibroblasts (**b**). (**c**) Mitochondrial DNA copy number in fibroblasts from 3 HD and 3 P assessed by real time PCR. (**d, e**) Relative mRNA levels of transcription factors involved in mitochondrial DNA replication (**d**) and electron transport chain (ETC) components and uncoupler protein 2 (UCP2)(**e**) in fibroblasts from 3 HD and 3 P assessed by real time PCR. (**f**) Western blot of UCP2 and β-actin expression in fibroblasts from 3 HD and 3 P. (**g, h**) Analysis of mitochondrial degradation (mitophagy) by co-staining of fibroblasts from a HD and P with MitoTracker (green) and LysoTracker DND-99 (red). Nuclei are counterstained with DAPI (blue). Insets on the right show single cells at higher magnification. Representative images (left) and Mander's coefficients of co-localization of MitoTracker and LysoTracker in cells from 3 HD and 3 P (right). (**h, i**) The mitochondrial membrane potential (MMP) was analyzed in TMRM-loaded HD (blue) and P (red) fibroblasts using single cell imaging. Averaged traces of 3 HD and 3 P (**h**) and analysis of basal MMP (**i**) before oligomycin treatment on a scale from 0 to 1, where 1 is set to maximum MMP measured after hyperpolarization of mitochondria with 1 μM oligomycin, and 0 is defined as minimum MMP after depolarization with 1 μM FCCP. (**j**) Proton

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pumping rate of the ETC measured as the MMP hyperpolarization rate after application of oligomycin in (**h**). (**k-m**) Mitochondrial superoxide production and ETC electron transport rate. Fibroblasts from 3 HD (blue) and 3 P (red) were loaded with MitoSOX and analyzed by flow cytometry before and at different time points after application of 1 μM oligomycin. Normalized MitoSOX signal (ΔMFI) over time (**k**), basal MitoSOX signal (ΔMFI) before application of oligomycin (**l**) and electron transport rate by the ETC as the rate of superoxide production after oligomycin treatment (**m**). (**n-p**)NADH levels and TCA cycle turnover. The redox state of cells was measured by NADH autofluorescence in fibroblasts from 3 HD (blue) and 3 P (red) using time-lapse microscopy. Relative minimum (0) and maximum (1) NADH fluorescence values were determined by treating cells with 1 μM FCCP to deplete NADH and 1 μM antimycin A plus 100 nM rotenone to maximize NADH levels. Averaged relative NADH autofluorescence over time on a scale from 0 to 1 (**n**), basal NADH levels after background subtraction (**o**) and TCA cycle turnover (**p**), which was calculated as the rate by which cells replenished the NADH pool upon antimycin A plus rotenone treatment. (**q, r**) Relative mRNA levels of *CPT1B* (**q**) and *ACADVL*(**r**) in fibroblasts from 3 HD and 3 P after culture in high-glucose medium or oleic acid (OA)-BSA-containing medium. All values represented as bar graphs are normalized to mean values obtained in HD fibroblasts unless indicated otherwise. All values are mean ± SEM. Statistical analysis was performed using Student's t-test. * $p<0.05$.

Figure 3. SOCE controls basal and fatty acid challenge-induced lipolysis. (**a**) Analysis of lipolysisin fibroblasts from 3 healthy donors (HD) and 3 patients (P) with loss-of-function mutations in *STIM1* or *ORAI1*. After 24 hour culture in high-glucose or oleic acid-BSA-containing media, lipolysis was induced by starvation in medium containing 1 mM glucose in the presence or absence of isoproterenol (10 μM). Free glycerol levels were measured after 24 hours. (**b-d**) Analysis of lipolysis in NIH3T3-L1 cells transduced with dominant negative ORAI1-E106Q (red) or empty vector control (blue). (**b**) SOCE was analyzed after passive depletion of ER Ca^{2+} stores with thapsigargin (TG) and re-addition of 1 mM Ca^{2+}Ringer's solution. (**c, d**) After 24 h culture of NIH3T3-L1 cells in medium containing high glucose (**c, d**), oleic acid-BSA (**c**) or palmitic acid-BSA (**d**), lipolysis was induced by starvation in medium containing 1mM glucose in the presence or absence of isoproterenol (10 μM). Free glycerol levels were measured after 6 h. All values represented as bar graphs in a, c and d are normalized to mean values obtained in HD fibroblasts or empty vector-transduced NIH3T3-L1 cells after culture in high glucose medium. All values are mean ± SEM. Statistical analysis was performed using Student's t-test. * $p<0.05$.

Figure 4. Increased lipophagy protects SOCE deficient cells from lipotoxicity. (**a**) Immunoblot for LC3 in fibroblasts from healthy donors (HD) and patients (P) with loss-of-function mutations in *STIM1* or *ORAI1*treated or not with lysosomal protease inhibitors (PI). (**b**) LC3-II steady-state levels (left) and autophagic flux (right) calculated by densitometric analysis of immunoblots.(**c**) Images of HD and P fibroblasts lentivirally transduced with the tandem fluorescent LC3 autophagy reporter mCherry-GFP-LC3. Merged and single channels are shown. Nuclei were stained with DAPI. (**d**) Average number of autophagic vacuoles (AV), autophagosomes (APG) and autolysosomes (AUT) per cell section calculated by imaging cells shown in panel c by high content microscopy. Average of 3 HD and 3 P are shown. n >1,200 cells per condition in duplicate in 9 fields each.(**e, f**)Long-lived protein degradation in 3 HD and 3 P fibroblasts. Total protein degradation rates (**e**) and percentage of lysosomal protein degradation and

macroautophagy-dependent degradation (3-methyladenine (3-MA) sensitive) calculated in cells supplemented with the respective inhibitors (**f**). (**g**) HD and P cells challenged with oleic acid and stained with Bodipy (green) to detect neutral lipid stores and with an antibody against LC3 (red). Nuclei were stained with DAPI. Representative micrographs of merged channels. Boxed area at higher magnification (middle) and detail of lipid droplets (right) show co-localized pixels in white. (**h, i**) Percentages of lipid droplets (LD) positive for LC3 ($LC3^+$) (**h**) and autophagic vacuoles (AV) positive for Bodipy (**i**) calculated from images similar to the ones in panel **g**. (**j-k**) ATG7 knockdown causes lipotoxicity. (**j**) Immunoblot for ATG7 in HD and P fibroblasts lentivirally transduced with an shRNA against a scrambled sequence (-) or ATG7 (+). (**k**) Viability of transduced cells after exposure to regular culture conditions (10mM glucose), 1 mM oleic acid (OA) or 0.5 mM palmitic acid (PA) for 24 hours. All values are mean ± SEM. Differences with HD are significant for * $p<0.05$.

Figure 5. Fatty acid-induced SOCE controls expression of PPARα and PGC-1α. (**a**) Intracellular Ca^{2+} levels in fibroblasts from healthy donors (HD, blue) and patients with loss-of-function mutations in *STIM1* or *ORAI1* (P, red) measured by single-cell Ca^{2+} imaging upon challenge with 0.5 mM oleic acid-BSA in RPMI1640 medium. Traces represent the averages of 3 HD and 3 P. At least 20 cells per donor were analyzed. (**b, c**) Relative mRNA levels of PGC-1α and PPARαin fibroblasts from 3 HD and 3 P (**b**) and in NIH3T3-L1 cells expressing the dominant negative mutant ORAI1-E106Q or an empty vector as control (**c**) after culture in high-glucose or oleic acid (OA)-containing medium. (**d**) Relative mRNA levels of PGC1αin NIH3T3-L1 cells cultured for 6 h in high-glucose medium alone (left bar) or stimulated in addition with thapsigargin (TG) to induce SOCE. The calcineurin inhibitor FK506 (1 μM), adenylate cyclase inhibitor SQ22,536 (100μM) or CAMK inhibitor KN93 (10 μM) were added for 6 h as indicated. Alternatively cells were cultured in OA-containing medium for 6 h. PGC-1αmRNA levels were determined by real time PCR. (**e, f**) Relative mRNA levels of PGC-1α, PPARα, STIM1 and STIM2 determined by real time PCR in the heart (**e**) and liver (**f**) of *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ *UBC-ER*^{T2}*-Cre* mice and Cre-negative littermates 5 days after the first injection of tamoxifen. (**g, h**) Relative mRNA levels of PGC-1α, PPARα, STIM1 and ORAI1 in the heart (**g**) and liver (**h**) of WT mice that were fed a normal diet or fasted for 24 h. All values represented as bar graphs are normalized to the mean value obtained in WT untreated samples. All values are mean ± SEM. Differences with HD are significant for * $p<0.05$.

Supplementary Figures, Tables and Videos

Supplementary Figure 1. MitoKeima reporter detects increased incidence of mitochondria undergoing lysosomal degradation (mitophagy) in SOCE-deficient cells. Representative images of fibroblasts from healthy donors (HD) and patients (P) with loss-of-function mutations in *STIM1* or *ORAI1*that were transiently transfected with a plasmid expressing MitoKeima. Images were taken after excitation at 543 nm, which preferentially excites Keima at an acid pH. The 3 images on the right show examples of cells at higher magnification. Nuclei are stained with DAPI. The bar graph shows the MitoKeima^{543nm}, -positive cell area relative to total cellular area calculated from the average of values in 3 HD and 3 P. All values are mean ± SEM. Differences with HD are significant for * p<0.05.

Supplementary Figure 2. Reduced expression of electron transport chain (ETC) proteins is associated with attenuated mitochondrial respiration. **(a)** O_2 consumption measurements in intact fibroblasts from one control subject (Co1) and two patients (P1, P2). Respiration rates were measured under basal conditions (basal respiration) and in the presence of the mitochondrial uncoupler FCCP (maximum respiration). Data are represented as mean ± SD. Statistically significant comparisons (two-tail, unpaired t-test) are indicated with ns (not significant), * (p<0.05), ** (p<0.01), ***(p < 0.001). Data are representative of 2 independent experiments. **(b)** SDS-PAGE and Western blot analysis of whole cell lysates from two controls (Co1, Co2) and two patient (P1, P2) fibroblast cell lines. Membranes were probed with antibodies directed against mitochondrial proteins and β actin as a loading control. Shown are representative images of at least 3 independent experiments. **(c)** BN-PAGE of DDM solubilized mitochondria from control (Co2) and patient (P1) fibroblasts followed by Western blot analysis. Membranes were probed with antibodies against NDUFA9 for complex I (CI) and the supercomplex (CICIII2), SDHA for complex II (CII), Core2 for complex III ($CIII_2$), COX4 for complex IV (CIV) and subunit of complex V (CV). Data are representative of 3 independent experiments.

Supplementary Figure 3. Mitochondria ultrastructure in heart from*Orai1^{R93W}*-knock-in mice. (**a**). Representative electron micrographs of heart sections from wild-type (WT) and *Orai1^{R93W}*knock-in (KI) mice. The image on the right shows the boxed area in the middle panel at a higher magnification to illustrate focal severe mitochondrial alterations (very low cristae density). (**b**) Electron micrographsfrom the same animals at higher magnification showing that even in areas from KI hearts where mitochondria show matrix densities comparable to wild type, they are aberrantly distributed. Arrows indicate lipid droplets (LD) of higher abundance in the heart of *Orai1^{R93W}* mice. (**c, d**) Morphometric analysis was used to calculate the average mitochondrial area, perimeter, larger and shorter diameter (**c**) and mitochondria size distribution (**d**) in four electron micrograph sections from 3 WT and 3 KI mice. The average number of mitochondria counted per micrograph was 20.All values represent the mean ± SEM. Differences compared to WT are significant for * p< 0.05. The morphometric analysis shows that the mitochondria size and length were significantly lower in *Orai1^{R93W}* compared to WT mice.

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Supplementary Figure 4. Increased expression of autophagy-related proteins in SOCE-deficient cells. (**a**) Representative immunoblots for the indicated proteins in fibroblasts from healthy donors (HD) and patients (P) with loss-of-function mutations in *STIM1* or *ORAI1* grown in regular medium (RPMI 1640, 10% FBS, 10 mM glucose). (**b-e**) Levels of TFEB (**b**), conjugated Atg5/12(**c**), p62 (**d**) and LAMP-1 (**e**) calculated from the densitometric quantification of immunoblots (including the one shown in panel a) from 3 HD and 3 P. Values are expressed as folds the average value in HD that was given an arbitrary value of 1.All values are mean ± SEM. Differences with HD are significant for * p<0.05.

Supplementary Figure 5. Ultrastructure of autophagic compartments and lipid droplets in the heart of*Orai1*^{R93W} knock-in mice. (**a**) Representative electron micrographs of heart sections from wild-type (WT) and *Orai1*^{R93W} knock-in (KI) mice. Images on the right show examples of autophagic vacuoles at higher magnification and at different stages of maturation (autophagosomes: double membrane with identifiable cargo indicated by yellow arrows; autolysosomes: single membrane or partial double membrane, with clear content or highly condense amorphous material indicated by a black arrows. Red arrows: lipid droplets (LD) with morphological features compatible with lipophagy. (**b, c**) Morphometric analysis was used to calculate the average number of autophagic vacuoles (AV) per section (**b**) and the percentage of autophagosomes (APG) and autolysosomes (AUT) (**c**) in micrographs including the ones shown in (**a**) from 3 WT and 3 KI mice. (**d-f**) Enhanced lipophagy in the heart of *Orai1*^{R93W} mice. (**d**) LD with morphological features compatible with lipophagy observed with more frequency in KI mice samples (as shown by the red arrows in panel a). All images shown are of KI mice. (**e ,f**) Morphometric analysis was used to calculate the average number of LD per section (**e**) and the percentage of total LD that are intact or show morphological features compatible with degradation by lipophagy (**f**). At least 6-8 micrographs from 3WT and 3 KI mice were counted. All values represent the mean ± SEM. Differences with WT are significant for * p< 0.05.

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Supplementary Figure 6. PPAR agonists neutralize autophagy upregulation in SOCE-deficient cells. Fibroblasts from healthy donors (HD) and ORAI1-deficient patients (P) were lentivirally transduced with the tandem fluorescent LC3 autophagy reporter mCherry-GFP-LC3. Nuclei were stained with DAPI. Shown are merged images of all three channels. (**a**) Representative images of fibroblasts from one HD and one ORAI1-deficient patient (P) that were left untreated or incubated with 5 μM of the PPARγ agonist Pioglitazone or 10 μM of the PPARα agonist WY14643 for 24 hours. Nuclei were stained with DAPI. **(b)** Representative images of NIH3T3-L1 mouse fibroblasts stably transfected with an empty vector or a vector expressing a dominant negative mutant of ORAI1 (E106Q) and subjected to the same treatment as in panel (**a**). The bar graphs in (a) and (b) show autophagic flux calculated as the average number of mCherry only-labeled autolysosomes per cell. All values are mean ± SEM. Differences with HD are significant for * p<0.05.

Supplementary Figure 7. SOCE-deficient fibroblasts upregulate PGC-1β expression. Relative mRNA levels of PGC1βin fibroblasts from 3 healthy donors and 3 patients with loss-of-function mutations

in *STIM1* or *ORAI1*. Fibroblasts were cultured in RPMI 1640 (10% FBS, 10 mM glucose). Values represented by bar graphs are mean ±SEM. Differences with HD are significant for * p<0.05.

Supplementary Figure 8. Model of the regulation of lipid metabolism by SOCE. Extracellular fatty acids induce SOCE, which is crucial for metabolic reprogramming of cells when fatty acid nutrients are in abundance. Fatty acid transporters coupled to phospholipase have been described to result in Ca^{2+} release from the ER via IP3Rs and activation of STIM1, which binds to and opens ORAI1 Ca^{2+} channels. The subsequent Ca^{2+}influx activates Ca^{2+} dependent adenylate cyclases and calcineurin initiating signaling cascades that result in the transcription of PGC-1α and PPARα genes. PGC-1α and PPARα are master transcriptional regulators of genes involved in lipid metabolism. In the absence of SOCE, cells fail to upregulate PGC-1α and PPARα upon fatty acid challenge, resulting in (1) impaired expression of enzymes involved in fatty acid transport, beta-oxidation and electron transport chain function in mitochondria, (2) impaired lipolysis of triglycerides and (3) accumulation of lipid droplets. Lipophagy is initiated in SOCE-deficient cells as a protective mechanism against lipotoxicity in conditions of fatty acid challenge when PPARα fails to initiate conventional lipid metabolism.

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Supplementary Table 1. Impaired electron transport chain complex I and IV activity in skeletal muscle biopsies from patients with loss-of-function mutations in *ORAI1*. Respiratory chain enzyme activities in the skeletal muscle of three SOCE-deficient patients with loss-of-function mutations in *ORAI1* (Patient 1: ORAI1 p.L194P; Patient 2: ORAI1 p.G98R; Patient 3: ORAI1 p.G98R) were performed by two independent clinical laboratories as previously described (Gempel et al., 2007; Kirby et al., 2007). Biochemical analysis of respiratory chain enzyme activity in skeletal muscle of 2 out of 3 patients revealed a decreased activity of one or more enzymes.

Supplementary Table 2. List of oligonucleotide primers used for quantitative real-time PCR.

Supplementary Video 1. Acute deletion of *Stim1* and *Stim2* genesin mice causes muscular dystonia and myclonus-like symptoms. *Stim1$^{fl/fl}$Stim2$^{fl/fl}$ UBC-ER^{T2}-Cre^{+}*mice were injected with 1 mg tamoxifen / 20 g body weight (and their *Cre^{-}*littermates, Supplementary Video 2) by i.p. injection daily for 5 consecutive days; mice were analyzed on day X after the first tamoxifen injection. Myoclonus-like symptoms were induced by gently lifting mice by their tails and maintaining them in an inverted position.

Supplementary Video 2.*Stim1$^{fl/fl}$Stim2$^{fl/fl}$ UBC-ER^{T2}-Cre^{-}*(WT littermates) mice injected with tamoxifen as described in the legend to Supplementary Video 1 lack dystonia and myoclonus-like symptoms.

Supplementary Video 3. Altered gait, uncoordinated muscle movements, shaking and dystonic posturing in a *Stim1$^{fl/fl}$Stim2$^{fl/fl}$ UBC-ER^{T2}-Cre^{+}*mouse (with 2 red stripes its tail) after tamoxifen treatment

as described in the legend to Supplementary Video 1. No pathological signs were observed in tamoxifen-treated *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ *UBC-ER*^{T2}*-Cre*$^{-}$ (WT) littermates.

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