MCUR1 is an essential component of mitochondrial Ca\(^{2+}\) uptake that regulates cellular metabolism

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Ca\(^{2+}\) flux across the mitochondrial inner membrane regulates bioenergetics, cytoplasmic Ca\(^{2+}\) signals and activation of cell death pathways\(^1\)–\(^11\). Mitochondrial Ca\(^{2+}\) uptake occurs at regions of close apposition with intracellular Ca\(^{2+}\) release sites\(^1\)–\(^11\), driven by the inner membrane voltage generated by oxidative phosphorylation and mediated by a Ca\(^{2+}\) selective ion channel (MiCa; ref. 15) called the uniporter\(^1\)–\(^18\) whose complete molecular identity remains unknown. Mitochondrial calcium uniporter (MCU) was recently identified as the likely MCU-dependent mitochondrial Ca\(^{2+}\) uptake channel (MiCa; ref. 15) called the uniporter\(^1\)–\(^18\) whose complete molecular identity remains unknown. Mitochondrial calcium uniporter (MCU) was recently identified as the likely uniporter channel complex required for mitochondrial Ca\(^{2+}\) uptake. MCU-dependent mitochondrial Ca\(^{2+}\) uptake (Supplementary Fig. S1a–d). Of the 45 genes, RNAi against only one, coiled-coil domain containing 90A (CCDC90A), a previously undescribed protein hereafter referred to as mitochondrial calcium uniporter regulator 1 (MCUR1), was found to markedly disrupt oxidative phosphorylation, lowers cellular ATP and activates AMP kinase-dependent pro-survival autophagy. Thus, MCUR1 is a critical component of a mitochondrial uniporter channel complex required for mitochondrial Ca\(^{2+}\) uptake and maintenance of normal cellular bioenergetics.

To identify genes important for mitochondrial Ca\(^{2+}\) uptake, we performed a directed human RNA-mediated interference (RNAi) screen of 45 mitochondrial membrane proteins in HEK293T cells predicted or reported to be integral mitochondrial inner membrane proteins, or with previously proposed roles in mitochondrial Ca\(^{2+}\) regulation (Supplementary Tables S1–S3). At 96 h after transfection with pools of three short interfering RNAs (siRNAs) targeting each gene, cytoplasmic (Fluo-4) and mitochondrial (rhod-2) [Ca\(^{2+}\)]\(_i\) were simultaneously imaged by confocal microscopy\(^2\)–\(^3\). To rapidly elevate cytoplasmic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\); Fig. 1a) to trigger mitochondrial Ca\(^{2+}\) uptake, either a Ca\(^{2+}\) ionophore, ionomycin, was employed at a concentration that enhanced plasma membrane Ca\(^{2+}\) permeability while leaving mitochondrial membranes intact, or stimulation by an inositol trisphosphate (InsP\(_3\))-linked agonist was used (Supplementary Fig. S1a–c and Movie S1). siRNA against most genes had no effect on mitochondrial Ca\(^{2+}\) uptake (Fig. 1b). Some siRNAs caused modest changes, including those targeted to MICU1 (ref. 21), CHCHD3, TMEM186, LETM1 (ref. 26) and SL25A23. Although MCU was not included in the original screen, we validated the screening methodology by demonstrating that MCU knockdown abrogated mitochondrial Ca\(^{2+}\) uptake (Supplementary Fig. S1d).

To confirm this result, five lentiviral short hairpin RNA (shRNA) constructs that targeted different regions of the MCUR1 gene (Supplementary Table S2) were used to create stable HeLa and HEK293T cell lines with MCUR1 knocked down by 42 to 87% among different clones by quantitative real time PCR (qRT-PCR; Fig. 1d,e).

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Two HEK293T cell clones with 80 and 87% MCUR1 messenger RNA knockdown (shHe1 and shHe2, respectively) and two HeLa cell clones with 74 and 87% mRNA knockdown (shHe1 and shHe2, respectively), with >75% and 95% reduced protein expression, respectively (Fig. 1f) were used for more detailed analyses of mitochondrial Ca\(^{2+}\) uptake and cellular bioenergetics. Stable knockdown of MCUR1 in HEK293T cells was achieved using two different shRNA constructs, shHe1 and shHe2, which resulted in >75% and 95% reduced protein expression, respectively. These cells were used to study the effects of MCUR1 knockdown on mitochondrial Ca\(^{2+}\) uptake, cellular bioenergetics, and overall cell function.

[Figure 1: RNAi screen identifies MCUR1 as a regulator of mitochondrial Ca\(^{2+}\) uptake. (a,b) Changes in HEK293T cell cytoplasmic (a) and mitochondrial (b) Ca\(^{2+}\) in response to ionomycin (2.5 μM) were simultaneously measured by Fura-4 and rhod-2 imaging, respectively. Each bar represents one target gene silenced with pooled siRNA. (c) qRT-PCR of MCU, MCUR1 and MICU1 mRNA from mouse tissues (n = 3; mean ± s.e.m.). (d) qRT-PCR of MCUR1 mRNA from HEK293T cell clones (n = 3; mean ± s.e.m.). KD, knockdown. Neg shRNA, negative shRNA. (e) qRT-PCR of MCUR1 mRNA from HEK293T cell clones and of rescued MCUR1 mRNA levels in shHe2 clone cells (n = 3; mean ± s.e.m.). The same lentiviral shRNAs were used to generate shHK4 and shHe1 and shHK5 and shHe2, respectively. (f) MCUR1 protein expression levels (top) and densitometric analysis (bottom left) (n = 3; ± s.e.m.). Bottom right, Flag-tagged MCUR1 protein expression in clone shHe2 cells reconstituted with shRNA-resistant MCUR1 cDNA plasmid, WB, western blot. (g,h) Representative images from movies of HEK293T negative shRNA or shHK5 cells showing cytosolic (green) and mitochondrial (red) Ca\(^{2+}\) before, during and after ionomycin exposure. Scale bar, 20 μm. (i-p) Cytoplasmic (green) and mitochondrial matrix (red) Ca\(^{2+}\) responses in HEK293T (i-l) and HeLa (m-p) cells challenged with ionomycin or histamine (100 μM), respectively (n = 3). (l) Wild-type HEK293T cells. (j) Cells expressing negative shRNA. (k) Clone shHK5 (n = 4). (l) Quantification of peak rhod-2 fluorescence. **p < 0.01 (mean ± s.e.m.). (m) HeLa cells expressing negative shRNA. (n) Clone shHe2. (o) Clone shHe2 re-expressing MCUR1 (n = 3). (p) Quantification of peak rhod-2 fluorescence. *p < 0.05, **p < 0.01 (mean ± s.e.m.). (q) [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_m\) signals evoked by ATP (100 μM) and thapsigargin (1 μM) monitored simultaneously using fura-2/AM and mitopericam (ICam 490), respectively in control (left) and MCUR1 knockdown (right) HeLa cells. [Ca\(^{2+}\)]\(_i\) is calibrated in nanomolar concentrations (black), whereas mitopericam fluorescence is inversely normalized to baseline (F₀/F; red). (r) Summary mean [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_m\) peaks during ATP stimulation (negative shRNA n = 29; MCUR1 knockdown n = 36 cells). *p < 0.05 (mean ± s.e.m.). (s) Increase in bath [Ca\(^{2+}\)] ([R\(_{bath}\)]) and [Ca\(^{2+}\)]\(_m\) (R\(_{pm} \)) signals in response to CaCl\(_2\) (1 μM) and InsP\(_3\) (IP\(_3\), 7.5 μM) addition in permeabilized cells. Uncropped images of blots/gels are shown in Supplementary Fig. S6.]

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cell clone shHK5 strongly abrogated the \([\mathrm{Ca}^{2+}]_m\) rise (Fig. 1h,k,l; see Supplementary Movie S1—negative shRNA, Supplementary Movie S2—shHK4 and Supplementary Movie S3—shHK5), in contrast to normal responses in wild-type cells (Fig. 1i) and cells expressing a negative shRNA (Fig. 1g,j,l). Histamine triggered similar \(\text{InsP}_3\)-mediated \([\mathrm{Ca}^{2+}]_m\) elevations in both negative shRNA (Fig. 1m) and MCUR1-knockdown HeLa cells (clone shHe2; Fig. 1n), whereas mitochondrial \(\text{Ca}^{2+}\) uptake was significantly diminished in MCUR1-knockdown cells (Fig. 1n,p). Although compartmentalized rhod-2 has been widely used to measure \([\mathrm{Ca}^{2+}]_m\) in intact cells (for example, refs 23–27), to ensure specificity of the fluorescent signal \([\mathrm{Ca}^{2+}]_m\) was also recorded by a \(\mathrm{Ca}^{2+}\)-sensing fluorescent protein, inverse pericam, genetically targeted to the mitochondria (mitopericam). These studies showed that the ATP-induced \([\mathrm{Ca}^{2+}]_m\) signal was selectively suppressed in intact MCUR1-knockdown HeLa cells (Fig. 1q,r). Furthermore, the \(\text{InsP}_3\)-induced \([\mathrm{Ca}^{2+}]_m\) rise was also attenuated in MCUR1-knockdown permeabilized cells (Fig. 1s). To confirm the target specificity of \(\text{MCUR1}\) shRNA, a rescue experiment was performed in HeLa shHe2 cells using a \(\text{MCUR1}\) complementary DNA with four silent point mutations in the shRNA target region and a Flag epitope (DDK tag). Stable expression of the rescue cDNA construct \(\text{MCUR1}\) contains two transmembrane helices, with a local amino-terminal fragment proximal to the first transmembrane helix. Furthermore, loss of the FLAG-tag by proteinase K treatment suggests that the C-terminal end of \(\text{MCUR1}\) also faces the cytosolic side. These results suggest that \(\text{MCUR1}\) plays an important role in mitochondrial \(\text{Ca}^{2+}\) uptake.

Mitochondrial \(\text{Ca}^{2+}\) uptake is driven primarily by the mitochondrial inner membrane voltage \(\left(\psi_m\right)\), maintained by the electron transport chain and oxidative phosphorylation. Knockdown of \(\text{MCUR1}\) did not alter \(\Delta \psi_m\) in intact or permeabilized HeLa cells (Supplementary Fig. S2e–g), nor did it alter mitochondrial DNA copy number (Supplementary Fig. S2h). Importantly, knockdown of \(\text{MCUR1}\) expression was without effect on the normal mitochondrial localization of \(\text{MCU}\) (Supplementary Fig. S2i). Notably, \(\text{MCU}\) mRNA and protein levels were upregulated in cells with \(\text{MCUR1}\) knocked down (Supplementary Fig. S2j–l). Thus, \(\text{MCUR1}\) is not required for \(\text{MCU}\) expression or localization. Collectively, these results suggest that \(\text{MCUR1}\) plays a regulatory role in \(\text{MCU}\)-dependent mitochondrial \(\text{Ca}^{2+}\) uptake.

To explore this further, the effects of \(\text{MCUR1}\) knockdown on mitochondrial \(\text{Ca}^{2+}\) uptake and \(\Delta \psi_m\) were examined in digitonin-permeabilized cells that were bathed in intracellular-like medium containing thapsigargin to prevent endoplasmic reticulum (ER) \(\text{Ca}^{2+}\) uptake, Fura2FF to monitor \([\mathrm{Ca}^{2+}]_m\) in the medium and JC-1 to monitor \(\Delta \psi_m\). In response to \(\text{Ca}^{2+}\) added to the medium, energized mitochondria rapidly reduce medium \([\mathrm{Ca}^{2+}]_m\) by Ru360- and CCCP-sensitive uptake that causes inner membrane depolarization (Fig. 2a,f). In permeabilized HEK293T cells expressing negative shRNA, mitochondria rapidly cleared multiple pulses of externally added 10 \(\mu\mathrm{M} \text{Ca}^{2+}\) (Supplementary Fig. S3a). In contrast, both \(\text{MCUR1}\)-knockdown clones shHK4 and shHK5 demonstrated nearly complete inhibition of external \(\text{Ca}^{2+}\) clearance (Supplementary Fig. S3b). \(\text{MCUR1}\) clone shHK2 exhibited an intermediate ability to take up external \(\text{Ca}^{2+}\), correlated with the intermediate level of \(\text{MCUR1}\) knockdown in these cells (Supplementary Fig. S3b). The addition of 10 \(\mu\mathrm{M} \text{Ca}^{2+}\) boluses triggered rapid mitochondrial \(\text{Ca}^{2+}\) uptake that caused small inner membrane depolarization in negative shRNA HeLa cells (Fig. 2a). In contrast, cells with \(\text{MCUR1}\) knocked down (Fig. 2b,c) demonstrated strong inhibition of mitochondrial \(\text{Ca}^{2+}\) uptake without depolarization. Reconstitution of \(\text{MCUR1}\) in HeLa clone shHe2 cells restored mitochondrial \(\text{Ca}^{2+}\) uptake and consequent inner membrane depolarization (Fig. 2d). To establish further that \(\text{MCUR1}\) facilitates mitochondrial \(\text{Ca}^{2+}\) uptake, HeLa cells stably overexpressing \(\text{MCUR1}\) were generated. \(\text{MCUR1}\)-overexpressing cells were able to clear more cytosolic \(\text{Ca}^{2+}\) pulses compared with negative shRNA HeLa cells (Fig. 2e), without altering CGP37157-sensitive Na\(^+\)/\text{Ca}^{2+}\) exchanger-mediated \(\text{Ca}^{2+}\) influx rate (Fig. 2j–l). To determine whether \(\text{MCUR1}\)-dependent mitochondrial \(\text{Ca}^{2+}\) uptake is mediated by \(\text{MCU}\), we used the MCU blocker Ru360. Ru360 inhibited mitochondrial \(\text{Ca}^{2+}\) uptake in plasma-membrane-permeabilized cells in response to bath addition of boluses of \(\text{Ca}^{2+}\) in cells overexpressing \(\text{MCUR1}\) as well as in control cells and cells with \(\text{MCUR1}\) knocked down (Fig. 2f–i). Furthermore, basal mitochondrial matrix \(\text{Ca}^{2+}\) was reduced in \(\text{MCUR1}\)-knockdown cells (Fig. 2m,n). Together, these data strongly implicate \(\text{MCUR1}\) in the mechanism of unipporter-mediated mitochondrial \(\text{Ca}^{2+}\) uptake.

Co-expression of carboxy-terminal \(\text{GFP}\)-tagged \(\text{MCUR1}\) and \(\text{DsRed-Mito}\), or \(\text{C-terminal mRFP}\)-tagged \(\text{MCUR1}\) and \(\text{EYFP-Mito}\) confirmed the mitochondrial localization of \(\text{MCUR1}\) (Fig. 3a,b). The membrane localization of \(\text{MCUR1}\) was evaluated by sub-cellular fractionation followed by mitochondrial sub-fractionation. In western blots, anti-Flag antibody detected a band with the expected relative molecular mass of \(\text{MCUR1}\) (\(M_r \sim 40,000\)) that was highly enriched in HeLa cell mitoplasts (Fig. 3c). Most hydrophobic analyses suggest that \(\text{MCUR1}\) contains two transmembrane helices, with a \(\sim 60\)-residue amino terminus and a C terminus predicted to contain only a couple of amino acids projecting into the same compartment. The membrane topology of \(\text{MCUR1}\) was investigated by proteinase K treatment of plasma-membrane-permeabilized (digitonin) cells. Permeabilized cells were incubated with truncated \(\text{Bid}\) (t\(\text{Bid}\)) to selectively permeabilize the outer mitochondrial membrane. After outer mitochondrial membrane permeabilization, samples were challenged with proteinase K. Membrane fractions were solubilized and subjected to western blot analysis. A mitochondrial matrix protein, \(\text{HSP60}\), was protected from proteinase K digestion (Fig. 3d). In contrast, the inner mitochondrial integral membrane protein \(\text{OXA1}\) and \(\text{MCUR1}\) were cleaved (Fig. 3d). Proteinase K produced a truncated \(\text{MCUR1}\) fragment \(\sim 6\) kD smaller than the full-length protein, consistent with loss of the amino-terminal fragment proximal to the first transmembrane helix. Furthermore, loss of the FLAG-tag by proteinase K treatment suggests that the C-terminal end of \(\text{MCUR1}\) also faces the cytosolic side. These results suggest that the N and the predicted short C termini could be exposed to the inter-membrane space, consistent with the presence of two transmembrane spanning regions with most of the protein present in the matrix.

\(\text{MCU}, \text{MICU1}\) and \(\text{MCUR1}\) are targeted to mitochondria and regulate mitochondrial \(\text{Ca}^{2+}\) uptake. \(\text{MICU1}\) physically associates with \(\text{MCU}\) (refs 19,22). To determine whether \(\text{MCUR1}\) similarly interacts with \(\text{MCU}\), Flag-tagged \(\text{MCUR1}\) and GFP-tagged \(\text{MCU}\) were co-expressed and precipitated with Flag or GFP antibodies and immunoblotted with the reciprocal antibodies. \(\text{MCUR1}\) was able to pull down \(\text{MCU}\), and vice versa (Fig. 3e). In contrast, \(\text{MCUR1}\) and \(\text{MICU1}\)
Figure 2 | MCUR1 is required for Ru360-sensitive mitochondrial Ca\(^{2+}\) uptake but is independent of the mitochondrial Ca\(^{2+}\) efflux pathway. Digitonin-permeabilized HeLa cells bathed in intracellular-like solution containing thapsigargin (Tg) were loaded with the ΔΨm indicator JC-1 and the Ca\(^{2+}\) indicator Fura2FF, to which pulses of 10 μM Ca\(^{2+}\) were added before the addition of the mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydrazone). (a–e) Representative traces from three independent experiments depict simultaneous changes of bath [Ca\(^{2+}\)] and ΔΨm in cells expressing negative shRNA (Neg shRNA; a), clone shHe1 (b), clone shHe2 re-expressing MCUR1 (d) and HeLa cells stably overexpressing MCUR1 (e). Under similar conditions, 1 μM Ru360 was added before 10 μM Ca\(^{2+}\) pulses until the addition of CCCP. (f–i) Representative traces from three independent experiments depict simultaneous changes of bath [Ca\(^{2+}\)] and ΔΨm in negative shRNA cells (f), MCUR1-knockdown clone shHe1 (g) and shHe2 (h), and in control cells overexpressing MCUR1 (i). (j,k) Negative shRNA (j) and MCUR1-overexpressing (k) HEK293T cells were permeabilized with digitonin in intracellular-like medium containing thapsigargin and bath [Ca\(^{2+}\)] indicator Fura2FF, and then pulsed with 10 μM Ca\(^{2+}\). After mitochondrial clearance of bath Ca\(^{2+}\), Ru360 caused an elevation of bath [Ca\(^{2+}\)], indicating that steady-state bath [Ca\(^{2+}\)] after the pulse was maintained by a balance of MCU-mediated Ca\(^{2+}\) uptake and CGP37157 (10 μM)-sensitive Na\(^{+}\)-Ca\(^{2+}\) exchanger-mediated extrusion. CCCP was added as indicated. The solid line is the mean; shaded areas are ±s.e.m. (n = 3). (l) [Ca\(^{2+}\)]\(_{\text{efflux}}\) rate derived from j and k during the initial 60 s following Ru360 addition (NS; not significant; n = 3). (m) HEK293T cells stably expressing negative shRNA or MCUR1 shRNA (clone shHe2) and clone shHe2 re-expressing MCUR1 cells were permeabilized with digitonin in intracellular-like medium containing the bath [Ca\(^{2+}\)] indicator Fura2FF. CCCP was added as indicated. Traces show bath [Ca\(^{2+}\)] (μM). Solid lines are the mean; shaded regions are ±s.e.m. (n = 3). (n) Quantification of total mitochondrial Ca\(^{2+}\) released after CCCP addition. *P < 0.05, **P < 0.01 (mean±s.e.m.).
did not interact, although we could confirm the interaction of MICU1 and MCU (Fig. 3f). Immunoprecipitation of ectopically expressed coiled-coil domain containing inner mitochondrial membrane protein LETM1 failed to pull down MCU–Flag (Fig. 3g) whereas anti-Flag immunoprecipitation of MCU–Flag but not LETM1–Flag co-immunoprecipitated MCU–V5 (Fig. 3h). Furthermore, immunoprecipitation of MICU1, MCU or MICU1 did not pull down endogenous inner membrane proteins OXA1 or COXIV (Supplementary Fig. S4a). Of note, the results in Fig. 3e,f suggest that MCU exists in a complex with either MICU1 or MCU1, but not both simultaneously. To examine this further, we transiently expressed all three tagged proteins, and immunoprecipitated V5-tagged MICU1. MCU1 pulldown co-immunoprecipitated MCU but not MICU1 (Supplementary Fig. S4b), suggesting that the three
MCUR1 is essential for MCU-dependent mitochondrial Ca\(^{2+}\) uptake. (a) qRT-PCR of MCU mRNA from wild-type and stable MCU1-overexpressing HeLa cells that were transiently transfected with scrambled siRNA or MCU siRNA against MCU. **P < 0.001 (mean ± s.e.m.).** (b) [Ca\(^{2+}\)]\(_{m}\) responses to histamine (100 \(\mu\)M) in HeLa cells stably overexpressing MCU and in cells transiently transfected with scrambled siRNA or MCU siRNA, and in stable MCU1-overexpressing HeLa cells transfected with MCU siRNA. After 48 h of siRNA transfection, cells were loaded with rhod-2 and [Ca\(^{2+}\)]\(_{m}\) responses were visualized by confocal microscopy. Solid lines are mean, shaded regions are ± s.e.m.; n = 3. (c) Quantification of peak rhod-2 fluorescence following histamine stimulation. *P < 0.05, **P < 0.001 (mean ± s.e.m.; NS, not significant; n = 3). (d) qRT-PCR of MCU mRNA from wild-type and MCU1-knockdown HeLa cells that were transiently transfected with MCU cDNA. **P < 0.01, ***P < 0.001 (mean ± s.e.m.; n = 3). (e) [Ca\(^{2+}\)]\(_{m}\) responses to histamine (100 \(\mu\)M) in wild-type and MCU1 (shHe2)-knockdown HeLa cells overexpressing MCU. Negative shRNA (Neg shRNA) and MCU1-shHe2 cells were used as controls. Solid lines are mean; shaded regions are ± s.e.m.; n = 3. (f) Quantification of peak rhod-2 fluorescence following histamine stimulation. ***P < 0.001 (mean ± s.e.m.; n = 3).

To confirm this further, we examined the dependence of MCU1-mediated mitochondrial Ca\(^{2+}\) uptake on MCU. We first confirmed the requirement of MCU for histamine-induced mitochondrial Ca\(^{2+}\) uptake by examining cells with MCU knocked down (Fig. 4). Histamine-stimulated mitochondrial Ca\(^{2+}\) uptake was markedly enhanced in cells stably overexpressing MCU1 (Fig. 4b,c). However, knockdown of MCU (Fig. 4a) strongly blunted MCU1-enhanced Ca\(^{2+}\) uptake (Fig. 4b,c). We next examined whether overexpression of MCU could restore mitochondrial Ca\(^{2+}\) uptake in cells with MCU1 knockdown. MCU overexpression enhanced mitochondrial Ca\(^{2+}\) uptake in wild-type but not in MCU1-knockdown HeLa cells (Fig. 4d-f). Thus, both MCU and MCU1 expression are required for efficient mitochondrial unipporter-mediated Ca\(^{2+}\) uptake.

Mitochondrial unipporter uptake of constitutively released Ca\(^{2+}\) from the ER is essential for regulation of optimal cellular bioenergetics by providing sufficient reducing equivalents to support oxidative phosphorylation\(^{28}\). Absence of this Ca\(^{2+}\) transfer results in reduced O\(_{2}\) consumption and ATP levels and activation of AMP kinase (AMPK) that activates pro-survival macroautophagy\(^{28}\). As a distinct approach to evaluate the role of MCU1 in mitochondrial Ca\(^{2+}\) uptake, we measured bioenergetic parameters in control and MCU1-knockdown HEK293T and HeLa cells. The AMP/ATP ratio was enhanced by ∼2-fold in stable MCU1-knockdown cells when compared with negative shRNA HeLa cells, and this enhancement was rescued by re-expression of shRNA resistant MCU1 (Fig. 5a). In both HeLa and HEK293T cells, stable (Fig. 5 and Supplementary Fig. S5a–d) or transient (Supplementary Fig. S5e–j) knockdown of MCU1 reduced basal O\(_{2}\) consumption rates (Fig. 5b,c and Supplementary Fig. S5a,b,g,i), reflecting diminished oxidative phosphorylation; caused constitutive activation of AMPK (Fig. 5d,f and Supplementary Fig. S5c,f,i); and induced macroautophagy (Fig. 5e,g and Supplementary Fig. S5d,e,h). These phenotypes were not observed in negative shRNA cells. Importantly, they were reversed to control levels by re-expression of MCU1 (Fig. 5a–e). Of note, they are similar to the effects elicited by stable knockdown of MCU in HeLa cells (Supplementary Fig. S5k). These bioenergetic abnormalities observed in cells with strongly reduced MCU1 expression are highly reminiscent of those induced proteins do not exist in one complex under the conditions of our experiments. Together, these results demonstrate that MCU1 physically associates with MCU and is necessary for MCU-mediated mitochondrial Ca\(^{2+}\) uptake.
Figure 5 MCUR1 is required for the maintenance of cellular bioenergetics. (a) AMP/ATP ratios in stable HeLa cell lines stably expressing negative shRNA (Neg shRNA), MCUR1 shRNA (clone shHe2) or shHe2 with MCUR1 re-expressed. **P < 0.01 (mean ± s.e.m.; n = 3). (b) OCR consumption rates (OCR) in stable HeLa cells expressing irrelevant shRNA, clone shHe2, and clone shHe2 re-expressing MCUR1, exposed sequentially to oligomycin (i), FCP (ii) and rotenone plus myxothiazol (iii). (c) Basal and maximal OCR consumption rates in cells as described in b. *P < 0.05 (mean ± s.e.m.; n = 3). (d) Western blot of phosphorylated and total AMPK (top) and densitometric analysis (bottom) in stable HeLa lines expressing negative shRNA or MCUR1 shRNA (clone shHe2) and clone shHe2 re-expressing MCUR1. *P < 0.05, **P < 0.01 (mean ± s.e.m.; n = 3). (e) Western blot of LC3 or tubulin in stable HeLa lines expressing negative shRNA or MCUR1 shRNA (clone shHe2) and clone shHe2 re-expressing MCUR1 (top) and quantification of LC3-II/LC3-I + LC3-II (bottom) expressed as fold increase over levels in cells expressing irrelevant shRNA (clone shHe2) and clone shHe2 rescue Neg shRNA (clone shHe2 rescue Neg shRNA; NS). Uncropped images of blots/gels are shown in Supplementary Fig. S6.

by inhibition of InsP$_3$ receptor (InsP$_3$R)- and uniporter-dependent ER Ca$^{2+}$ transfer to mitochondria$^{29}$. The metabolic effects of InsP$_3$R and uniporter inhibition were previously observed to be non-additive$^{29}$. Although autophagy activation observed in stable MCUR1-knockdown HeLa cells was slightly potentiated by xestospongin B inhibition of InsP$_3$R activity (Fig. 5g; as was also the case in stable MCU knockdown cells; Supplementary Fig. S5k), activation of AMPK (HeLa; Fig. 5f and Supplementary Fig. S5f; HEK293T; Supplementary Fig. S5f) and autophagy (HeLa: Supplementary Fig. S5h; HEK293T, Supplementary Fig. S5e) by transient MCUR1 knockdown were not potentiated. These independent results also suggest that MCUR1 is an important component of the molecular machinery associated with the mitochondrial uniporter-mediated Ca$^{2+}$ uptake mechanism.

Our results demonstrate that the previously unstudied MCUR1 is essential for mitochondrial uniporter-mediated Ca$^{2+}$ uptake. In the absence of sufficient MCUR1 expression, mitochondrial Ca$^{2+}$ uptake is strongly blunted in both stimulated and basal conditions. This results in compromised cellular bioenergetics as a consequence of diminished oxidative phosphorylation that results in the activation of pro-survival autophagy. The effects of MCUR1 knockdown on Ca$^{2+}$ uptake and mitochondrial bioenergetics were observed here in two cell lines, suggesting that MCUR1 plays a general role in regulating mitochondrial Ca$^{2+}$ uptake. Inhibition of mitochondrial Ca$^{2+}$ uptake by MCUR1 knockdown did not cause mis-localization or lower expression of MCU, suggesting that MCUR1 plays a direct role in uniporter-mediated Ca$^{2+}$ uptake, possibly by a direct interaction with MCU that is required for MCU to function as the uniporter channel pore. Identification of MICU1 and MCUR1 as regulators of Ca$^{2+}$ uptake suggests that the mitochondrial Ca$^{2+}$ channel may consist of a complex of proteins associated with a Ca$^{2+}$ permeable pore subunit, probably MCU (refs 19,20). Enhanced total Ca$^{2+}$ uptake in cells with MCU overexpressed may be caused by increased MCU activity, although further studies are needed to understand the MCUR1 role in mitochondrial Ca$^{2+}$ buffering. Reconstitution of purified MCU into planar lipid bilayers was associated with the appearance of small conductance Ca$^{2+}$ channels$^{20}$. However, the properties were not completely similar to those of the uniporter recorded in situ by patch-clamp electrophysiology of mitoplasts$^{15}$, nor were they recorded under physiological ionic conditions. Thus, although MCU was shown to form a Ca$^{2+}$ channel in the absence of
other proteins in vitro, our results suggest that it requires MCUR1 to function in mitochondrial membranes. The discovery of MCUR1 as an integral component of the mitochondrial Ca\(^{2+}\) uptake machinery provides a new target for regulation of Ca\(^{2+}\) signalling related to signal transduction, bioenergetics, and cell survival and death.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper*

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**AUTHOR CONTRIBUTIONS**

K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., and M.M. analysed the results. G.H. and G.C. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work. K.M., M.M. and J.K.F. designed the project. K.M., C.C., P.D., H.C.C., K.M.I., P.M., J.Y., M.M., T.G., G.C. and R.M. performed the experimental work.

**COMPETING FINANCIAL INTERESTS**

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**METHODS**

**Cell lines.** HeLa (ATCC® CCL-2) and HEK293T/17 (ATCC® CRL 11268) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with l-glutamine supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C and 5% CO₂. Human primary fibroblasts (CF9) were obtained from the Coriell Institute for Medical Research and cultured in DMEM with l-glutamine supplemented with 15% FBS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C and 5% CO₂. RNAi. A custom Silencer Select Human siRNA Library (Ambion) was developed on the basis of a bioinformatic analysis performed by selecting candidates according to the following parameters: predicted/reported mitochondrial localization as integral inner membrane proteins; previously proposed roles in mitochondrial Ca²⁺ regulation; and mitochondrial-targeted but unstudied candidates. An RNAi screen against 45 mitochondrial candidate genes was carried out in HEK293T cells using the custom-made Silencer Select Human siRNA Library (Ambion). Stable knockdown of MCU1 in HeLa and HEK293T cells was achieved by transducing lentiviruses carrying shRNAs targeting MCU1 (Openbiosystems). HeLa or HEK293T cells were grown in 6-well plates coated with a mixture of three siRNAs (Custom Silencer Select Human siRNA Library, Ambion) to each gene at 50 nM using TransIT-siQUEST (MIR 2115, Mirus Bio LLC). For some genes, RNAi used pools of four distinct proprietary siRNAs (SMARTpool, Dharmacon; 50 nM) using DharmaFECT 1 (Dharmacon). As controls, non-targeting siRNA duplets (Dharmacon) were employed. Cells were used 72 h post-transfection. For MCU siRNA transfection, HeLa cells were transfected with pools of two distinct siRNAs (MCU-1 5′-GCCAGACGACGACAUUU-3′; MCU-2 5′-GGGAAUUGACAGAGGUUCCDIT-3′, Dharmacon; 50 nM) using DharmaFECT 1. Cells were used 48 h post-transfection.

**MICE-TISSUE gene expression analysis.** For gene expression analysis of MCU, MCU1 and MCU2 in mouse tissues, skeletal muscles (tibialis anterior), brain, heart, lung, liver, kidney, large intestine, small intestine, thymus, bone marrow, spleen and lymph nodes (auxiliary and mesenteric) were removed from age-matched (22–24 g) adult male C57BL/6 mice. Total RNA was extracted using Trizol reagent (Invitrogen) as per the manufacturer’s instructions. Total RNA (1 µg) was reverse transcribed with ABI high-capacity cDNA synthesis kit. qrtPCR reactions were performed with gene-specific Solaris qPCR assay kit (Ambogene) as per the manufacturer’s instructions. Solaris qPCR Gene Expression Assay for mouse gene CCDC109A (MCU) consisted of forward primer: 5′-ATCCAGGCCCAAGTGTTC-3′; and probe: 5′-CCAATGAGAAGCAGCCAG-3′. HeLa or HEK293T cells (3 × 10⁴ per well) grown on 0.1% gelatin-coated coverslips in six-well dishes were transfected with a mixture of three siRNAs (Custom Silencer Select Human siRNA Library, Ambion) to each gene at 50 nM using TransIT-siQUEST (MIR 2115, Mirus Bio LLC). For some genes, RNAi used pools of four distinct proprietary siRNAs (SMARTpool, Dharmacon; 50 nM) using DharmaFECT 1 (Dharmacon). As controls, non-targeting siRNA duplets (Dharmacon) were employed. Cells were used 72 h post-transfection.

**mtDNA isolation, subfractionation and MCU1 topology analysis.** Mitochondria-containing pellet fractions were subjected to immunoblotting against antibodies against VDAC (Mitosciences, MSA03; dilution 1:1,000), cytochrome oxidase subunit II (MiToSciences, MS405; dilution 1:1,000) and Flag (Sigma, #F3165; dilution 1:1,000). For transfection experiments, HeLa cells (~5 × 10⁴) were permeabilized with 40 µg ml⁻¹ digitonin in 500 µl of intracellular-like medium (ICM) (120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Tris–HEPES at pH 7.2 and 2 mM MgATP (ref. 51)) buffer for 10 min. To evoke outer mitochondrial membrane permeabilization, digitonin-permeabilized cells were incubated with tBid (50 nM) for 10 min (ref. 32). Outer mitochondrial membrane permeabilization was confirmed by the cytosolic appearance of cytochrome c (BD-Pharmpingen, #556433, dilution 1:1,000). After outer mitochondrial membrane permeabilization, with or without tBid, treated samples were incubated with proteinase K for 10 min. Mitochondria-containing pellet fractions were subjected to immunoblotting against anti-HSP60 (Abcam #ab110312; dilution 1:500), anti-OX1 (Sigma #SAB408614; dilution 1:500), anti-Flag (Sigma #F3165; dilution 1:1,000) and anti-MCU1 (Aviva Systems Biology #ARP4777; dilution 1:1,000) antibodies.

**Inubomatoblot analysis and immunoprecipitation.** Antibodies were from Cell Signaling Technology (Phospho-x1-AMPK (Thr 172) and L3C), Millipore (α-1-AMPK), ZYMED Laboratories (β-tubulin; dilution 1:1,000) and Amersham (secondary antibodies conjugated with peroxidase). The reagents FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), oligomycin, myxothiazol and rotenone were from Sigma. Protein extracts were prepared from HeLa clone shHe2 rescue cells. Proteins were separated in 4–12% gel and transferred to PVDF membranes using iBLot (Invitrogen), probed with anti-FLAG monoclonal antibody (Sigma, #F3165; dilution 1:1,000) and rabbit anti-mouse IgG (H+L) HRP conjugate (Bio-Rad) for 1 h and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). For analysis of AMPK and LC3, cells were prepared and analysed as described previously²⁶. Chemiluminescence detection used a series of timed exposures to ensure densitometric analyses were performed at exposures within the linear range.

**Immunoblot analysis and immunoprecipitation.** Antibodies were from Cell Signaling Technology (Phospho-x1-AMPK (Thr 172) and L3C).

**Generation of stable shRNA knockdown cell lines.** Five different lentiviruses carrying shRNAs targeting different regions of the target gene were generated with four silent point mutations in the human generic shRNA constructs (Openbiosystems), psPAX2 and pMD2.G (Addgene, as described²⁴). HeLa or HEK293T cells (5 × 10⁴ per well) grown in 6-well plates were transfected with lentivirus, selected with puromycin (2 mg ml⁻¹) 48 h post-transfection for 6–10 days and expanded. Knockdown was assessed by qPCR. Note that knockdown cells were used to generate shHK4 and shHe1, and shHK5 and shHe2, respectively.

**MCUR1 shRNA rescue and MCU1 overexpression.** An MCU1 rescue construct resistant to MCUR1 shRNA knockdown was created with four silent point mutations in the MCUR1 shRNA targeting region (ORIGENE). MCU1-knockdown cells were transfected with MCU1 rescue construct and 48 h post-transfection, selected with 200 µg ml⁻¹ G418 sulphate, hiCASP2 and DDM2-Flag-tagged MCUR1 cDNA was stably overexpressed in HeLa cells and selected with 500 µg ml⁻¹ G418 sulphate.

**qrtPCR analysis.** Total RNA was isolated from wild-type and negative shRNA stable cells using RNeasy Minikit (Qiagen). Total RNA (1 µg) was reverse transcribed with Verso cDNA synthesis kit (Ambogene). qrtPCR reactions were performed with gene-specific Solaris qPCR assay kit (Ambogene) for human MCU1 and MCU; forward primer: 5′-CTTCGCTTGACCAAGGAC-3′ and reverse primer: 5′-TGAGTACAAAACTCCGTTGC-3′; and forward primer: 5′-GTCATTGTCACCTAAGCACTT-3′, reverse primer: 5′-TGGAGCAGACGACGACAA-3′ and probe: 5′-TCTATTTACAGATGTTG-3′, respectively. Reactions were performed in triplicate and normalized to gpdh. Relative gene expression was calculated with untreated samples as a calibrator using 7300 Real Time PCR system RQ study software 1.4 (Applied Biosystems).

**Mitochondrial isolation, subfractionation and MCU1 topology analysis.** HeLa cells (10 × 10⁴) stably expressing C-terminal Flag-tagged MCU1 were collected, washed with ice-cold mitochondrial isolation buffer and mitochondria were isolated as previously described²⁶. The mitochondrial pellet was resuspended in digitonin (1:2%) containing isolation medium for 5 min under constant stirring. Samples were centrifuged at 14,000 × g (10–20 fixed-angle rotor) for 10 min and supernatant and pellet were collected separately. The crude mitochondria (35% of the total) were suspended in sucrose (1.2%) containing isolation medium and centrifuged at 50,000 × g (type 70 Ti rotor) for 60 min. The pellet fraction was used as the outer mitochondrial membrane fraction²⁶. These fractions (mitochondria, outer mitochondrial membrane and mitoplasts) were subjected to immunoblotting with antibodies against VDAC (Mitosciences, MSA03; dilution 1:1,000), cytochrome oxidase subunit II (Mitosciences, MS405; dilution 1:1,000) and Flag (Sigma, #F3165; dilution 1:1,000). For topology studies, HeLa cells (~5 × 10⁴) were permeabilized with 40 µg ml⁻¹ digitonin in 500 µl of intracellular-like medium (ICM) (120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Tris–HEPES at pH 7.2 and 2 mM MgATP (ref. 51)) buffer for 10 min. To evoke outer mitochondrial membrane permeabilization, digitonin-permeabilized cells were incubated with tBid (50 nM) for 10 min (ref. 32). Outer mitochondrial membrane permeabilization was confirmed by the cytosolic appearance of cytochrome c (BD-Pharmpingen, #556433, dilution 1:1,000). After outer mitochondrial membrane permeabilization, with or without tBid, treated samples were incubated with proteinase K for 10 min. Mitochondria-containing pellet fractions were subjected to immunoblotting against anti-HSP60 (Abcam #ab110312; dilution 1:500), anti-OX1 (Sigma #SAB408614; dilution 1:500), anti-Flag (Sigma #F3165; dilution 1:1,000) and anti-MCU1 (Aviva Systems Biology #ARP4777; dilution 1:1,000) antibodies.
Simultaneous measurement of cytoplasmic and mitochondrial Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{m}). HeLa/HEK293T/human primary fibroblasts were grown on 25-mm glass coverslips for 48 h and were loaded with 2 \mu M rhod-2-AM (30 min) and 5 \mu M Fura2FF (30 min) in extracellular medium as described previously\textsuperscript{24}. Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37 \degree C and imaged. After 1 min of baseline recording, agonist (histamine (100 \mu M); ionomycin (2.5 \mu M); thrombin (500 mU)) was added and confocal images were recorded every 3 s (510 Meta; Carl Zeiss) at 488- and 561-nm excitation using a x63 oil objective. Images were analysed and quantified using ImageJ (NIH) and custom-made software (Spectralyzer).

For pericam experiments, cells were transiently transfected with plasmid DNA encoding mitochondrial-matrix-targeted inverse pericam (44 \mu g ml\textsuperscript{-1}) using Lipofectamine 2000 for 24 h. Before recording, cells were pre-incubated in a serum-free extracellular medium (ECM: 121 mM NaCl, 5 mM Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 4.7 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, and 10 mM glucose, at pH 7.4) containing 2% BSA and were loaded with fura-2-AM for measurements of [Ca\textsuperscript{2+}]\textsubscript{i}, as described previously\textsuperscript{25}. For permeabilized-cell experiments, transfected cells were washed with a Ca\textsuperscript{2+}-free ECM containing 100 \mu M EGTA/TRIS and transferred to the imaging chamber in 1 ml ECM. Plasma membrane permeabilization was carried out using 40 \mu g ml\textsuperscript{-1} saponin at 35 \degree C for 5 min. The cells were then washed with fresh ECM supplemented with 2 mM succinate/EGTA/1.5 \mu M fura-2-free acid. Fluorescence wide-field imaging of [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{m} was carried out using a back-illuminated electron-multiplied charge-coupled device (CCD) camera (ProEM 1024 \times 1024, Princeton Instruments), fitted to a Leica DMI 6000B inverted epifluorescence microscope. For recording fura-2 and inverse pericam, 340/30 nm, 380/20 nm and 490/20 nm excitation filters were used with a 500 nm beam splitter and a 540/50 nm emission filter and an image triplet was obtained every 2 s. Fura-2 ratios were calibrated in nanomolar concentrations, whereas the inverse pericam fluorescence at each time was normalized to the initial fluorescence (F/0).

Simultaneous measurement of mitochondrial membrane potential (\Delta \Psi\textsubscript{m}) and Ca\textsuperscript{2+} uptake in permeabilized cells. Before permeabilization, cells were washed in an extracellular-like Ca\textsuperscript{2+}-free buffer (120 mM NaCl, 5 mM KCl, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 0.2 mM MgCl\textsubscript{2}, 0.1 mM EGTA and 20 mM HEPES-NaOH, at pH 7.4) and stored on ice for at least 10 min. Following centrifugation, cells (10 \times 10\textsuperscript{6}) were transferred to an intracellular-like medium (permeabilization buffer: 120 mM NaCl, 10 mM Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES-TRIS, at pH 7.2) containing 2% BSA and 100 \mu M succinate/EGTA. The cell suspension supplemented with succinate (2 \mu M) was placed in a fluorimeter and permeabilized by gentle stirring. Fura2FF (0.5 \mu M) was added at 0 s, and JC-1 (800 nm) at 20 s. Fluorescence signal was monitored in a temperature-controlled (37 \degree C) multiwavelength-excitation dual-wavelength-emission spectrofluorometer (Delta RAM, Photon Technology International) using 490-nm excitation/535-nm emission for the monomer, 570-nm excitation/595-nm emission for the J-aggregate of JC-1 and 340-nm/380-nm for Fura2FF (ref. 33). Ten-nanomolar Ca\textsuperscript{2+} pulses were added and the \Delta \Psi\textsubscript{m} and Ca\textsuperscript{2+} uptake were monitored simultaneously. CCIPC was added at 1,000 s as a control. Traces indicate the ratio of the JC-1 ratio and Fura2FF ratio from at least three independent experiments.

Quantification of adenine nucleotides. Nucleotides were extracted from HeLa cells using perchloric acid, neutralized and frozen for subsequent HPLC analysis\textsuperscript{34}. AMP and ATP in extracted samples were quantified using ion-pair reverse-phase HPLC, with a C18 RP column, under isocratic elution conditions in 200 mM phosphate, 5 mM tetrabutylammonium phosphate and 3% acetonitrile.

Mitochondrial DNA quantification. Total HeLa cell DNA was isolated by SDS–proteinase K digestion and 2 \mu g DNA digested by BamHI. Biological triplicate samples for each treatment group were resolved by electrophoresis through a 0.6% agarose gel in 0.5 \times TBE. Gels were processed and transferred by capillary action. Membrane was ultraviolet crosslinked and probed with a random primed radiolabelled probe against the nuclear 18S gene\textsuperscript{35} and signal determined by photo-stimulated luminescence. Membrane was re-probed using PCR-amplified human mitochondrial DNA (mtDNA), and the relative abundance of mtDNA to nuclear mtDNA calculated.

Oxygen consumption. The oxygen consumption rate was measured at 37 \degree C in an XF24 extracellular analyser (Seahorse Bioscience) as described previously\textsuperscript{26}. Cells were sequentially exposed to oligomycin (1 \mu M), FCCP (300 nM) and rotenone (100 nM) plus myxothiazol (100 nM). Every point in traces represents the average of 10 different wells. In preliminary studies of each cell type, three concentrations of cells were seeded. Linear relationships between O\textsubscript{2} consumption and the number of cells seeded were observed. The cell number that provided a nearly confluent uniform monolayer was chosen for study. Before and at the end of each experiment, the uniformity of the monolayer was evaluated by differential interference contrast microscopy.

Presentation of data and statistics. All graphs were produced using either Sigmaplot or GraphPad Prism software. Unless indicated, all recordings are representative of the mean fluorescence value of all cells per field, indicative of three or more independent experiments (n = 3 or more). Data from multiple experiments were quantified to determine the peak n-fold change or percentage change of arbitrary fluorescence units, expressed as mean ± s.e.m. Differences between the groups were analysed using Student’s t-test. Differences in means among multiple data sets were analysed using one-way analysis of variance with the Kruskal–Wallis test followed by pairwise comparison using the Dunn test. A P value less than 0.05 was considered significant in all analyses.

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CORRIGENDUM

MCUR1 is an essential component of mitochondrial Ca\textsuperscript{2+} uptake that regulates cellular metabolism

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Figure S1 Cytosolic and mitochondrial [Ca2+] and mitochondrial membrane potential responses to ionomycin. (a) [Ca2+]c (Fluo-4) and [Ca2+]m (rhod-2) responses to 2.5 μM ionomycin in HeLa cells imaged by confocal microscopy and analyzed by methods that excluded overlap of the two signals. (b) [Ca2+]c and [Ca2+]m responses to 2.5 μM ionomycin in HeLa cells pretreated with the mitochondrial uncoupler CCCP. Lack of effect of ionomycin on [Ca2+]m demonstrates that ionomycin at this concentration lacks direct effects on mitochondrial Ca2+ permeability. (c) Mitochondrial membrane potential (ΔΨm, measured by TMRE fluorescence) responses assessed by confocal microscopy of HeLa cells treated with different concentrations of ionomycin (2.5, 10 and 20 μM) and subsequent addition of a mitochondrial uncoupler. CCCP-induced depolarization demonstrates that lower [ionomycin] lack direct effects on mitochondrial permeability. (d) Inhibition of mitochondrial Ca2+ uptake in MCU siRNA treated HeLa cells.
Figure S2 Effects of MCUR1 knockdown on mitochondrial [Ca2+] uptake, ΔΨm, mtDNA copy number and MCU localization and expression. Mitochondrial [Ca2+] uptake is attenuated in MCUR1 ablated human primary fibroblasts. Changes in cytosolic and mitochondrial matrix [Ca2+] in response to thrombin (500 mU) were simultaneously measured by fluo-4 and rhod-2 imaging, respectively. [Ca2+]m responses to thrombin in (a) scrambled siRNA and (b) MCUR1 siRNA treated human fibroblasts. Quantification of rhod-2 (b) and fluo4 (d) peak fluorescence following thrombin stimulation. (for a and c, solid lines are mean; shaded regions are ± s.e.m.; n=3). ***P < 0.001 (mean ± s.e.m.; n=3). HeLa cells (Wild type, neg shRNA, MCUR1 shHe2 shRNA and MCUR1 shHe2 rescue) grown on glass bottom Petri-dishes were loaded with ΔΨm indicator TMRE (50 nM) to assess mitochondrial membrane polarization. Representative confocal images (e; scale bar: 20 µm)) and quantification (f) of ΔΨm maintenance (n=3). (g) HeLa cells (Neg shRNA, MCUR1 shHe2 shRNA and MCUR1 shHe2 rescue) grown on glass bottom Petri-dishes were loaded with TMRE (50 nM) and permeabilized with digitonin. Quantification of TMRE fluorescence intensity changes from cells imaged every 3 sec. CCCP was added at indicated time point. Entire sequence of TMRE fluorescence (a.u) is shown (mean ± s.e.m; n=3). (h) Relative mtDNA copy number in HeLa cell clones (n=3). (i) Co-localization of the mitochondrial protein TOM20 and MCU in stable MCUR1 knockdown HeLa cell clone shHe2. Scale bar: 15 µm. (j) MCU mRNA levels in WT HeLa cells and in stable lines expressing negative shRNA, MCUR1 shRNA (clone shHe2) and in clone shHe2 expressing shRNA-insensitive MCUR1. *P < 0.05, ***P < 0.001 (mean ± s.e.m.; n=6). (k) Western blot of MCU in stable HeLa lines. (l) Quantitation of MCU protein expression *P < 0.05, **P < 0.01 (mean ± s.e.m; n=5).
Figure S3 Kinetic analysis of mitochondrial Ca2+ uptake in permeabilized 293T cells. Cells were permeabilized with digitonin in presence of thapsigargin (Tg) and Fura2FF. 10 μM Ca2+ boluses were sequentially added to the medium before addition of the mitochondrial uncoupler CCCP (2 μM). (a) Cells stably expressing negative shRNA (control). (b) Cells expressing different MCUR1 target shRNA with different knockdown efficiencies.
**Figure S4** Specificity of MCUR1 interaction with MCU. (a) Immunoprecipitation of MCUR1-Flag, MCUR1-V5 or MCU-GFP failed to pull down inner membrane proteins OXA1 or COXIV. (b) Co-immunoprecipitation of MICU1-Flag with MCU-GFP but not with MCUR1-V5 in doubly or triply transiently transfected COS7 cells. (C) Immunoprecipitation of LETM1-Flag with Flag antibody failed to pull-down MCUR1-V5 in doubly transiently transfected COS7 cells. Representative of three independent experiments.
Figure S5 Bioenergetic deficits in cells with MCUR1 stably knocked down. 
(a-d) Bioenergetic deficits in 293T cells with MCUR1 stably knocked down. 
(a) O2 consumption rates (OCR) in stable 293T clone shHK5 or negative 
shRNA clone exposed sequentially to (a) oligomycin, (b) FCCP, and (c) 
rotenone plus myzothiazol. 
(b) Basal and maximal OCR in cells described 
in (a). *P < 0.05 (mean ± s.e.m.; n=4). 
(c) Western blot of phosphorylated 
and total AMPK (top) and densitometric analysis (bottom) in stable 293T 
cell lines expressing negative shRNA or MCUR1 shRNA (clone sheHe2) 
and clone ShHe2 re-expressing MCUR1. **P < 0.01, ns = not significant. 
(mean ± s.e.m.; n=5). 
(d) Western blot of LC3 or tubulin in stable HeLa 
lines expressing negative shRNA or MCUR1 shRNA (clone sheHe2) 
and clone ShHe2 re-expressing MCUR1 (top) and quantification of LC3-II/ 
(LC3-I + LC3-II) (bottom) expressed as fold increase over levels in cells 
expressing irrelevant shRNA. *P < 0.05, **P < 0.01 (mean ± s.e.m; n=5). 
(e-j) Bioenergetic deficits in 293T (e-g) and HeLa (h-j) cells with MCUR1 
transiently knocked down. (e and h) Western blot of LC3 or tubulin (top) 
and quantification of LC3-II/(LC3-I + LC3-II) (bottom) expressed as fold 
increase over levels in cells expressing negative shRNA in 293T (e) and 
HeLa (h) cells transiently transfected with pools of 4 irrelevant (Neg) or 
MCUR1-targeted siRNA in absence or after 30 min exposure to 5 μM XeB. 
**P < 0.01 (mean ± s.e.m; n=3). 
(f and i) Western blot of phosphorylated 
and total AMPK (top) and densitometric analysis (bottom) in 293T (f) 
and HeLa (i) cells transiently transfected with pools of 4 irrelevant (Neg) 
or MCUR1-targeted siRNA in absence or after 30 min exposure to 5 μM 
XeB. **P < 0.01 (mean ± s.e.m.; n=3). 
(g and j) Basal and maximal O2 
consumption rates in 293T (g) and HeLa (j) cells transfected 72 hrs earlier 
with pools of 4 irrelevant (Neg) or MCUR1-targeted siRNA. **P < 0.01 
(mean ± s.e.m; n=3). 
(k) Western blot of LC3 and tubulin in HeLa cells with MCU stably knockdown 
(bottom) and quantification (top) in presence or absence of Xestospongin 
B (XeB) expressed as fold increase over scrambled siRNA as control. *P < 
0.05, **P < 0.01 (mean ± s.e.m; n=3).
Figure S6 Full scans of Western blots. Each panel in each figure labeled to refer to data presented in main and supplemental figures.
Supplementary Information

Related to Figure 3c

Related to Figure 3d

Related to Figure 3g

Related to Figure 3h

Figure S6 continued
Figure S6 continued
Related to Supplementary Fig 2k

![Image of protein bands](image1)

Related to Supplementary Fig 5c, d, e, f, h, i & k

![Image of protein bands](image2)

Figure S6 continued
Related to Supplementary Fig 4a,b & C

Figure S6 continued
Supplementary Movie Legends

**Movie S1** Real time confocal imaging of cytoplasmic Ca2+ rise (Fluo-4 fluorescence; green) and mitochondrial Ca2+ uptake (Rhod2 fluorescence; red) in HEK293T cells stably expressing negative shRNA. Fluorescence intensity changes were imaged every 3 sec. Ionomycin added after 20th frame. Entire sequence of fluorescence is shown.

**Movie S2** Real time confocal imaging of cytoplasmic Ca2+ rise (Fluo-4 fluorescence; green) and mitochondrial Ca2+ uptake (Rhod2 fluorescence; red) in HEK293T cells stably expressing MCUR1 shHK4 shRNA. Fluorescence intensity changes were imaged every 3 sec. Ionomycin added after 20th frame. Entire sequence of fluorescence is shown.

**Movie S3** Real time confocal imaging of cytosolic Ca2+ rise (Fluo-4 fluorescence; green) and mitochondrial Ca2+ uptake (Rhod2 fluorescence; red) in HEK293T cells stably expressing MCUR1 shHK5 shRNA. Fluorescence intensity changes were imaged every 3 sec. Ionomycin added after 20th frame. Entire sequence of fluorescence is shown.

Table S1 List of siRNA used in screening experiments
A RNAi screen against mitochondrial candidate genes listed in the table was carried out in 293T cells using custom made Silencer Select Human siRNA Library (Ambion).

Table S2 List of lentiviral shRNA sequences used in the study
For stable knockdown of mitochondrial genes, cells were transduced with lentiviruses carrying shRNAs listed in the table.

Table S3 Order of samples used in screening related to Figure 1a and b.
Simultaneous measurements of changes in cytosolic and mitochondrial Ca2+ levels following agonist stimulation in siRNA transfected 293T cells were performed by confocal microscopy. The peak cytosolic (Fluo-4) and mitochondrial (rhod-2) fluorescence amplitude represented as % changes are provided in the table.