Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca\(^{2+}\) uniport

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Mitochondrial Ca\(^{2+}\) uptake is crucial for the regulation of the rate of oxidative phosphorylation\(^1\), the modulation of spatio-temporal cytosolic Ca\(^{2+}\) signals\(^2,3,4\) and apoptosis\(^5\). Although the phenomenon of mitochondrial Ca\(^{2+}\) sequestration, its characteristics and physiological consequences have been convincingly reported\(^6,7\), the actual protein(s) involved in this process are unknown. Here, we show that the uncoupling proteins 2 and 3 (UCP2 and UCP3) are essential for mitochondrial Ca\(^{2+}\) uptake. Using overexpression, knockdown (small interfering RNA) and mutagenesis experiments, we demonstrate that UCP2 and UCP3 are elementary for mitochondrial Ca\(^{2+}\) sequestration in response to cell stimulation under physiological conditions — observations supported by isolated liver mitochondria of Ucp2\(^{-/-}\)/Ucp3\(^{-/-}\) mice lacking ruthenium red-sensitive Ca\(^{2+}\) uptake. Our results reveal a novel molecular function for UCP2 and UCP3, and may provide the molecular mechanism for their reported effects\(^8-10\). Moreover, the identification of proteins fundamental for mitochondrial Ca\(^{2+}\) uptake expands our knowledge of the physiological role for mitochondrial Ca\(^{2+}\) sequestration.

Uncoupling proteins (UCPs) are embedded in the inner mitochondrial membrane and belong to the superfamily of mitochondrial ion transporters\(^11\). UCP1, which is preferentially expressed in brown adipose tissue, accounts for heat production by inducing a H\(^{+}\) leak that uncouples oxidative phosphorylation. In contrast, the physiological function of the family members UCP2 and UCP3, which have been identified in many tissues\(^11\), is still a matter of debate. Despite the fact that both proteins promote H\(^{+}\) influx in isolated mitochondria under specific conditions, their involvement in heat production has not been confirmed to date (for review see ref. 10). UCP2- and UCP3-related proteins also exist in ectothermic fish and plants, which do not require thermogenesis, emphasizing that additional functions of these UCPs may exist. Consistently, evidence has accumulated that points to their contribution in many cellular processes, such as mitochondrial free-radical production\(^12\), apoptosis\(^13\), the regulation of hormone secretion (UCP2)\(^14\), and glucose and fatty acid metabolism (UCP3)\(^15\). However, the reported smooth uncoupling by UCP2 and UCP3 (ref. 12) seems unlikely to account exclusively for such variety of functions. As, in contrast with H\(^{+}\), Ca\(^{2+}\) is known to control a large amount of biological processes, we assessed the involvement of UCP2 and UCP3 in mitochondrial Ca\(^{2+}\) homeostasis.

Initially, expression of UCPs was evaluated in endothelial cells. Using RT–PCR (Fig. 1A) and western blot analyses (Fig. 2a), the expression of UCP2 and/or UCP3, but not UCP1, could be verified in the human umbilical vein-derived cell line EA.hy926 (ref. 16; Fig. 1A), as well as in various human cell lines and short-term cultured human umbilical vein endothelial cells (see Supplementary Information, Fig S1a). After cloning and sequence verification, respective fluorescent fusion proteins of UCP2 (gi13259540) and UCP3 (gi13259544) were constructed and expressed in endothelial cells, where they revealed exclusive mitochondrial targeting (Fig. 1B). Subsequently, the consequences of overexpression of UCP2 or UCP3 on mitochondrial Ca\(^{2+}\) signalling were assessed in single cells expressing the mitochondrial-targeted Ca\(^{2+}\) sensor ratio-metric pericam\(^17\), which exhibited identical dynamics (that is, maximum, minimum; see Supplementary Information, Fig. S1c) regardless of the protein levels of the UCPs. Overexpression of the proteins alone did not affect basic parameters that may influence the ability of the mitochondria to sequester Ca\(^{2+}\) — such as the architectural organization of the mitochondria (Fig. 1B and see Supplementary Information, Fig. S1b, e), their motility (see Supplementary Information, Movie 1), matrix pH (see Supplementary Information, Fig. S1c) and membrane potential (see Supplementary Information, Fig. S1d), basal ATP production (see Supplementary Information, Fig. S1e) or focal contacts with the endoplasmic reticulum (see Supplementary Information, Fig. S1f). In line with these findings, basal Ca\(^{2+}\) concentration in the mitochondria ([Ca\(^{2+}\)]\(_{\text{mit}}\); see Supplementary Information, Table S1) and the endoplasmic reticulum, and histamine-induced endoplasmic reticulum Ca\(^{2+}\) release (see Supplementary Information, Fig. S1g) were not affected by overexpression of UCP2 or UCP3. Remarkably, despite identical cytoplasmic Ca\(^{2+}\) elevation in response to histamine in cells transiently overexpressing UCP2 or UCP3 (see Supplementary Information, Fig. S1h), mitochondrial Ca\(^{2+}\) sequestration was enhanced by 33 and 76%,
Figure 1 Expression of UCP orthologues and functional tests on Ca\(^{2+}\) carrier function of UCP2 or UCP3 in human endothelial cells. (A) RT–PCR of UCPs in EA.hy926 (EA) cells and human white adipose tissue (WAT). Methods and primer sequences are available shown in the Supplementary Information, Methods. (B) Targeting of fusion proteins of UCP2 and UCP3. a and d show the localization of the fusion constructs of UCP2 and UCP3; b and e the localization of mito-tracker orange; and c and f the colocalization in the respective cell. Mitochondria structure was visualized in endothelial cells transfected with mitochondria-targeted DsRed\(^{30}\) (g) or DsRed and UCP2 alone (h). Further information is shown in the Supplementary Information, Fig. S1b. For comparison with mitochondrial fragmentation, DsRed transfected cells were treated with 2 µM FCCP for 5 min (i). The scale bars represent 10 µm in a-f and 2 µm in g-i. (C) UCP2 and UCP3 overexpression increased mitochondrial Ca\(^{2+}\) uptake. Mitochondrial Ca\(^{2+}\) elevation in response to 100 µM histamine was measured with mitochondria-targeted ratiometric-pericam\(^{17}\) in intact endothelial cells overexpressing the sensor, either alone (control; \(n = 32\)) or together with UCP2 (\(n = 23\)) or UCP3 (\(n = 18\)). The asterisk indicates \(P < 0.05\) versus control. (D) UCP2 and UCP3 overexpression had no effect on histamine-induced changes in the mainly pH-sensitive wavelength of pericam (\(F_{485}\); data shown correspond to that shown in C). (E) UCP2 and UCP3 increased capacity, but not sensitivity, of mitochondrial Ca\(^{2+}\) uptake. Concentration response curves of histamine on mitochondrial Ca\(^{2+}\) elevation were obtained in single endothelial cells overexpressing mitochondria-targeted ratiometric pericam either alone (control, \(n = 6–12\)) or together with UCP2 (\(n = 6–12\)) or UCP3 (\(n = 6–12\)). The asterisk indicates \(P < 0.05\) versus control. The respective data on cytosolic Ca\(^{2+}\) concentration are shown in the Supplementary Information, Fig. S1k. (F) UCP2 and UCP3 increased mitochondrial Ca\(^{2+}\) uptake independently of mitochondrial pH, mitochondrial Na\(^{+}\)–Ca\(^{2+}\) exchanger or mitochondrial membrane potential. In single endothelial cells overexpressing UCP2 (\(n = 11\)) or UCP3 (\(n = 14\)) mitochondria were depolarized by 2 µM FCCP and 2 µM oligomycin and the mitochondrial Na\(^{+}\)–Ca\(^{2+}\) exchanger was inhibited by CGP 37157, as described previously\(^{4}\). The asterisk indicates \(P < 0.05\) versus control (\(n = 14\)). The error bars represent s.e.m.
Figure 2 UCP2 or UCP3 knockdown by siRNAs resulted in diminution and/or elimination of mitochondrial Ca\(^{2+}\) uptake. (a) siRNAs against UCP2 or UCP3 (see Supplementary Information, Fig. S2a) diminished expression of the respective protein, indicated by western blot analysis. The bars indicate the optical density of the respective band in lower panels, which are normalized according the corresponding β-actin band. Representative blots from three independent experiments are shown. (b) Silencing UCP2 and/or UCP3 expression attenuated and/or eliminated mitochondrial Ca\(^{2+}\) uptake. Mitochondrial Ca\(^{2+}\) sequestration in response to histamine was measured in single mitochondria-targeted ratiometric pericam-transfected endothelial cells that were cotransfected with control siRNA (see Supplementary Information, Methods), or with siRNAs against UCP2 and/or UCP3. Average curves are shown on the left (control, n = 16; UCP2 siRNA, n = 17; UCP3 siRNA, n = 17; and UCP2/3 siRNA, n = 22). The distribution of individual independent experiments is shown on the right. The bars indicate the mean value. The single asterisk indicates P < 0.05 versus control and the double asterisk indicates P < 0.05 versus cells transfected with UCP2 siRNA. The corresponding data on cytosolic free Ca\(^{2+}\) are provided in the Supplementary Information, Fig. S2b. (c) In single HeLa cells, which express UCP3 but not UCP2 (see Supplementary Information, Fig. S1a), only the UCP3 siRNA (n = 14) was suitable to prevent mitochondrial Ca\(^{2+}\) sequestration. The asterisk indicates P < 0.05 versus control (n = 12) and UCP2 siRNA (n = 9). (d) In single HeLa cells transfected with UCP3 siRNA (n = 10), overexpression of UCP2 rescued mitochondrial Ca\(^{2+}\) uptake, as monitored with cotransfected mitochondria-targeted ratiometric-pericam. The asterisk indicates P < 0.05 versus control (n = 11) and UCP2 + UCP3 siRNA (n = 10). (e) Overexpression of UCP2 rescued mitochondrial Ca\(^{2+}\) uptake in suspended digitonin-permeabilized HeLa cells that were transfected with UCP3 siRNA (n = 6). Calcium Green-5N (0.1 μM) was used to measure the reduction of extra-mitochondrial Ca\(^{2+}\) by mitochondria in digitonin-permeabilized and suspended HeLa cells, which were transiently transfected with either the vector alone (control), UCP3 siRNA or UCP2 + UCP3 siRNA. As indicated (arrows), 5 μM digitonin (Dig.), 90 nmol Ca\(^{2+}\) and 2 μM FCCP were added in the presence of 1 μM thapsigargin. The asterisk indicates P < 0.05 versus control (n = 6) and UCP2 + UCP3 siRNA (n = 6). The error bars represent s.e.m.

respectively (Fig. 1C). In contrast, overexpression of UCP2 or UCP3 did not affect changes in the primarily pH-sensitive pericam fluorescence at 485 nm (Fig. 1D). Notably, the kinetics of mitochondrial Ca\(^{2+}\) removal after agonist washout was slightly faster in UCP-overexpressing cells (control, −0.073 ± 0.030, n = 32; UCP2, −0.085 ± 0.020, n = 23, not significant (n.s.) versus control; UCP3, −0.108 ± 0.026 [1−(F\(_{\text{off}}/F_{0})/\text{min}]), n = 19, P < 0.05 versus control), thus, indicating that the enhanced mitochondrial Ca\(^{2+}\) accumulation during cell stimulation is most likely due to increased mitochondrial Ca\(^{2+}\) uptake, but not to a decreased mitochondrial Ca\(^{2+}\) extrusion. In line with these findings, overexpression of
UCP2 and UCP3 strongly increased mitochondrial Ca\(^{2+}\) sequestration on Ca\(^{2+}\) entry (see Supplementary Information, Fig. S1i).

Identical findings were obtained in HeLa and CHO-K1 cells that were stimulated with 100 μM histamine or 10 μM ATP, respectively (see Supplementary Information, Fig. S1i), suggesting that the reported feature of UCP2 and UCP3 represents a ubiquitous phenomenon. These findings are consistent with earlier reports that UCP3 overexpression increased mitochondrial susceptibility to Ca\(^{2+}\)-dependent opening of the permeability transition pore in permeabilized HEK293 cells\(^{13}\). However, in intact cells, overexpression of UCP2 or UCP3 did not initiate opening of a cyclosporin A-sensitive transition pore on cell stimulation (data not shown), possibly due to the intact mitochondrial environment, which limits excessive mitochondrial Ca\(^{2+}\) overload.

Remarkably, expression of UCP1 in endothelial cells that do not constitutively express this protein had no effect on basal mitochondrial Ca\(^{2+}\) content, and did not alter mitochondrial Ca\(^{2+}\) sequestration on cell stimulation (see Supplementary Information, Fig. S1k). These data further confirm the concept that UCP2 and UCP3 exhibit different physiological functions than UCP1, and indicate that strong expression of an inner mitochondrial protein does not mimic the effect of UCP2 and UCP3 on mitochondrial Ca\(^{2+}\) permeability.

To explore the contribution of UCP2 and UCP3 to mitochondrial Ca\(^{2+}\) sequestration, the impact of UCP overexpression on the sensitivity and capacity of the mitochondrial Ca\(^{2+}\) uptake machinery was assessed by measuring the concentration-response relationship to histamine. These experiments revealed that overexpression of UCP2 and UCP3 increased the capacity of mitochondrial Ca\(^{2+}\) uptake (Fig. 1E), whereas the affinity to histamine-induced cytosolic Ca\(^{2+}\) elevations, which were not altered by the UCP overexpression (see Supplementary Information, Fig. S1l), remained unaffected (EC\(_{50}\)-histamine: control, 7.73 (4.96–11.74) μM; UCP2, 5.35 (3.26–8.77) μM; UCP3, 4.40 (2.44–7.93) μM; Fig. 1E).

Under physiological conditions, the extent of Ca\(^{2+}\) actually sequestered by the mitochondria strictly depends on the mitochondrial membrane potential\(^5\). Thus, it seemed possible that, on cell stimulation, UCP2 and UCP3 affect the bioenergetic status of the mitochondria, which results in an augmented capability of mitochondria to take up Ca\(^{2+}\). To explore this possibility, mitochondria were completely de-electro- and/or depolarized using 2 mM carbonyl cyanide-4-trifluoromethoxyphenyl-hyrazone (FCCP; a concentration suitable for complete depolarization of the mitochondria; see Supplementary Information, Fig. S1m) and 2 μM oligomycin, thus providing a condition in which the uptake of Ca\(^{2+}\) into mitochondria is not energetic anymore and, therefore, exclusively follows the concentration gradient of this ion. Unsurprisingly, mitochondrial Ca\(^{2+}\) sequestration was largely reduced under these conditions and exhibited only approximately 20% of that obtained under control conditions (Fig. 1F). Nevertheless, overexpression of UCP2 or UCP3 strongly augmented mitochondrial Ca\(^{2+}\) uptake on maximal cell stimulation with 100 μM histamine and 15 μM 2,5-di-butylhydroquinone (BHQ), even under these depolarizing conditions (Fig. 1F). As these experiments were performed in the presence of 10 μM CGP 37157, an inhibitor of mitochondrial Na\(^+\)–Ca\(^{2+}\) exchanger (NCX\(_{\text{mito}}\)), the obtained amplification of mitochondrial Ca\(^{2+}\) uptake cannot be due to the reversed mode activity of NCX\(_{\text{mito}}\) and supports the concept that UCP2 and UCP3 inherently accomplish mitochondrial Ca\(^{2+}\) uptake.

Notably, in contrast with the H\(^+\) conductance of UCP2 and UCP3 — which was found only on exposure to products of reactive oxygen species (ROS) metabolism, such as hydroxynonenal and, perhaps, fatty acids\(^8\) — our data suggest that these UCP orthologues achieve Ca\(^{2+}\) sequestration into mitochondria without any additional pre-activation. Thus, a fundamental role for UCP2 and UCP3 in the phenomenon of mitochondrial Ca\(^{2+}\) sequestration is emphasized.

To challenge this hypothesis, loss-of-function experiments were conducted using selective siRNA against UCP2 and UCP3 (see Supplementary Information, Fig. S2a). Compared with non-transfected controls and siRNA controls, siRNAs against UCP2 and UCP3 strongly reduced expression of the respective protein (Fig. 2a). Remarkably, in cells that were transfected with siRNA against either UCP2 or UCP3, mitochondrial Ca\(^{2+}\) uptake in response to histamine was strongly reduced (Fig. 2b), whereas cytosolic Ca\(^{2+}\) elevation was not altered (see Supplementary Information, Fig. S2b). As the efficiency of siRNA knockdown of the respective proteins varies depending on multiple factors, the effect of siRNAs against UCP2 and UCP3 on mitochondrial Ca\(^{2+}\) sequestration was demonstrated by plotting the distribution of the effects achieved in the individual experiments (Fig. 2b). Consistent with our overexpression experiments (Fig. 1C), siRNA against UCP3 diminished mitochondrial Ca\(^{2+}\) signalling to a greater extent than silencing of UCP2, suggesting that UCP3 more efficiently sequesters Ca\(^{2+}\) into the mitochondria than UCP2. Remarkably, double transfection of endothelial cells with the siRNAs against UCP2 and UCP3 yielded further reduction of mitochondrial Ca\(^{2+}\) elevation. Two-thirds of cells cotransfected with siRNAs against UCP2 and UCP3 exhibited a reduction in mitochondrial Ca\(^{2+}\) signalling greater than 80% of that achieved in control cells (Fig. 2b). In addition, similar experiments were performed in HeLa cells that express UCP3 but not UCP2 (see Supplementary Information, Fig. S1a): transfection with siRNA against UCP2 had no effect on mitochondrial Ca\(^{2+}\) elevation in response to histamine, whereas in cells transfected with siRNA against UCP3, mitochondrial Ca\(^{2+}\) elevation was diminished, or even abolished, in comparison with the respective control (Fig. 2c). Moreover, mitochondrial Ca\(^{2+}\) uptake in intact (Fig. 2d) and permeabilized (Fig. 2e) HeLa cells that were transiently transfected with siRNA against UCP3, could be rescued by cotransfection with UCP2.

To further support our concept of an elementary contribution of these UCP isoforms to mitochondrial Ca\(^{2+}\) uniport, Ca\(^{2+}\) uptake experiments were performed in liver mitochondria (Fig. 3) isolated from Ucp2\(^{-/-}\) mice\(^{18}\) and their wild-type littermates. In liver, Ucp2 was found to represent the only UCP isoform\(^19\), and thus mitochondria from this tissue are suitable to facilitate determination of the consequence of Ucp2 knockout on the Ca\(^{2+}\) uptake of the organelle. In suspended mitochondria isolated from wild-type littermates, mitochondria exhibited a strong Ca\(^{2+}\) uptake from medium that was diminished by approximately 41% with 100 nM ruthenium red (Fig. 3a, c). In contrast, uptake of extra-mitochondrial Ca\(^{2+}\) by suspended mitochondria isolated from Ucp2\(^{-/-}\) mice was reduced by approximately 53% compared with that observed in wild-type mitochondria, whereas 100 nM ruthenium red had no further effect (Fig. 3b, c). The remaining Ca\(^{2+}\) uptake in mitochondria isolated from Ucp2\(^{-/-}\) mice, or in the presence of ruthenium red, may be due to alternative Ca\(^{2+}\) uptake pathway(s) under these conditions (for example, Ca\(^{2+}\)-H\(^{+}\) or Na\(^{+}\)–Ca\(^{2+}\) exchanger). Therefore, comparable data were obtained in single mitochondria isolated from wild-type and Ucp2\(^{+/-}\) mice, and further confirmed the complete lack of ruthenium red-sensitive Ca\(^{2+}\) uptake in liver mitochondria from Ucp2\(^{-/-}\) animals (Fig. 3d–g).
Figure 3 In isolated liver mitochondria from Ucp2<sup>−/−</sup> mice, no ruthenium red (RR)-sensitive Ca<sup>2+</sup> uniporter exists. (a) Ca<sup>2+</sup> uptake of suspended isolated liver mitochondria from wild-type (WT) animals was measured by monitoring the reduction of extra-mitochondrial Ca<sup>2+</sup> on application of 140 nmol Ca<sup>2+</sup> mg<sup>−1</sup> mitochondrial protein with Calcium Green-5N (0.1 µM). Experiments were performed in the absence (n = 7) and presence (WT + RR, n = 4; single asterisk indicates P < 0.05 versus WT) of ruthenium red, an established inhibitor of mitochondrial Ca<sup>2+</sup> uniporter (100 nM). (b) In suspended liver mitochondria isolated from Ucp2<sup>−/−</sup> mice, Ca<sup>2+</sup> uptake (measured as described in a) was reduced (Ucp2<sup>−/−</sup>; n = 7). The remaining Ca<sup>2+</sup> uptake was insensitive to ruthenium red (100 nM; Ucp2<sup>−/−</sup> + RR; n = 4), n.s. versus Ucp2<sup>−/−</sup>. (c) Kinetics of mitochondrial Ca<sup>2+</sup> uptake within 30 s after addition of Ca<sup>2+</sup> calculated from the data presented in a and b. The asterisk indicates P < 0.05 versus WT. (d) Fluorescence image of fura-2 loaded single mitochondria freshly isolated from mouse liver. The scale bar represents 10 µm. (e) In isolated single liver mitochondria from wild-type animals, mitochondrial Ca<sup>2+</sup> sequestration was measured in the absence (WT, n = 44) and presence (WT + RR, n = 22) of ruthenium red (1 µM) by mitochondrial fura-2. The asterisk indicates P < 0.05 versus WT. (f) Ca<sup>2+</sup> uptake in isolated single mitochondria from Ucp2<sup>−/−</sup> mice measured by mitochondrial fura-2 was reduced (Ucp2<sup>−/−</sup>, n = 34) compared with that obtained in the wild-type littermates. The remaining Ca<sup>2+</sup> uptake into the liver mitochondria of Ucp2<sup>−/−</sup> mice was not ruthenium red-sensitive (Ucp2<sup>−/−</sup> + RR, n = 21). n.s. versus Ucp2<sup>−/−</sup>. (g) Ruthenium red-sensitive mitochondrial Ca<sup>2+</sup> uptake in single mitochondria freshly isolated from wild-type and Ucp2<sup>−/−</sup> (Ucp2<sup>−/−</sup>, asterisk indicates P < 0.05 versus WT) mice calculated from data in e and f. The error bars represent s.e.m (a-f) or indicate 95% confidence intervals at the times shown (g).
Expression of UCP2 and UCP3 in the yeast _Saccharomyces cerevisiae_ did not affect mitochondrial membrane potential and Ca$^{2+}$ sequestration, which was insensitive to ruthenium red (see Supplementary Information, Fig. S3). These data indicate that in freshly isolated single mitochondria of these yeast strains, no ruthenium red-sensitive Ca$^{2+}$ uptake exists and that UCP2 or UCP3 alone are not suitable to establish this phenomenon in this heterologous environment.

Although UCP2 and UCP3, which are approximately 72% homologous, share approximately 58% protein homology with UCP1 (see Supplementary Information, Fig. S4a), there is a wide consensus that

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Figure 4 Mutagenesis of UCP2 and UCP3 resulted in loss of Ca$^{2+}$ function of the proteins and provided dominant-negative UCP homologues. (a) Sequence alignment and identification of specific domains common for UCP2 and UCP3, but not UCP1. Additional information is shown in the Supplementary Information. (b) Using the ROBETTA full-chain protein structure prediction server (http://robetta.bakerlab.org), the protein structure of UCP2 and UCP3 was predicted to form a channel-like structure. As indicated, the mutation was introduced in a helix that is located on the matrix side of the inner mitochondrial membrane and results in shortening of the helix loop and loss of polarity. (c) Functional consequences on mitochondrial Ca$^{2+}$ uptake in single endothelial cells transiently expressing mitochondria-targeted ratiometric-pericam overexpressing mutated UCP2 (n = 25) or mutated UCP3 (n = 15). The asterisk indicates P <0.05 versus control (n = 17). (d) Mutated UCP2 failed to rescue mitochondrial Ca$^{2+}$ uptake in single HeLa cells transfected with UCP3 siRNA. HeLa cells were transfected with either a non-functional control siRNA (n = 14), UCP3 siRNA (n = 9) or UCP3 siRNA together with mutated UCP2 (UCP2mut + UCP3 siRNA, n = 9). Asterisk indicates P <0.05 versus control siRNA.
these orthologues do not exhibit a thermogenic function like UCP1. Thus, it was expected that the domain that is crucial for the Ca\textsuperscript{2+}-conduction of UCP2 and UCP3 would be localized in regions with high homology between UCP2 and UCP3, but not with UCP1. Using sequence-based annotation analysis, the protonated A-T-E-E domain of UCP2 and UCP3 in the second inter-membrane loop was identified as differing from the polar A-T-T-E sequence of UCP1 (Fig. 4a). It is predicted that this domain forms a helix that is localized at the matrix side of the inner mitochondrial membrane (Fig. 4b). Mutagenesis of this domain (Fig. 4a) slightly shortened the respective helix, but no other effect on the structure of the protein was predicted (Fig. 4b). The localization of UCP2 and UCP3 mutants did not differ from that of their non-mutant analogues (see Supplementary Information, Fig. 5b).

In functional experiments, overexpression of the mutants of UCP2 or UCP3 in endothelial cells failed to result in increased mitochondrial Ca\textsuperscript{2+} sequestration, but counteracted the constitutive Ca\textsuperscript{2+} uptake and allowed only a reduced and transient Ca\textsuperscript{2+} elevation compared with control cells (Fig. 4c). In HeLa cells, which express UCP3 but not UCP2 (see Supplementary Information, Fig. S1a), the UCP2 mutant failed to rescue mitochondrial Ca\textsuperscript{2+} uptake if UCP3 expression was knocked down with siRNA, and diminished the activity of constitutive UCP3 for Ca\textsuperscript{2+} sequestration (Fig. 4d). These data suggest that, despite their correct targeting, mutated UCP2 and UCP3 exhibit very little or no functional activity for mitochondrial Ca\textsuperscript{2+} uptake. In addition, as the mutated UCP2 interfered with constitutive (functional) UCP3 in HeLa cells, and given our findings that overexpression of UCP2 or UCP3 alone was suitable for elevating mitochondrial Ca\textsuperscript{2+} uptake, we speculate that these proteins form homo- and hetero-multimers to contribute to the mitochondrial Ca\textsuperscript{2+} uniporter, which becomes inactive if a mutated UCP2 or UCP3 is co-assembled.

As mitochondrial Ca\textsuperscript{2+} uptake has been shown to trigger agonist-induced ATP synthesis in mitochondria\textsuperscript{4}, agonist-induced mitochondrial ATP production can be used as an indirect measure of mitochondrial Ca\textsuperscript{2+} sequestration. Therefore, to investigate the physiological consequences of UCP2 and UCP3 availability, mitochondrial ATP synthesis was measured in cells expressing either UCP2 or UCP3. Notably, if these UCP orthologues exhibited H\textsuperscript{+} conductance and/or uncoupling under physiological conditions, agonist-induced mitochondrial ATP production would be reduced. If, on the other hand, the physiological function of these UCPs is the result of mitochondrial Ca\textsuperscript{2+} uniporter, we speculate that these proteins form homo- and hetero-multimers to contribute to the mitochondrial Ca\textsuperscript{2+} uniporter, which becomes inactive if a mutated UCP2 or UCP3 is co-assembled.

We have described a previously unknown physiological role for UCP2 and UCP3. For the first time, two proteins with known sequence are characterized as fundamental for mitochondrial Ca\textsuperscript{2+} uniport. Although, it remains unclear whether or not UCP2 and UCP3 represent the channel proteins responsible for mitochondrial Ca\textsuperscript{2+} sequestration, the characterization of these proteins as fundamental contributors to mitochondrial Ca\textsuperscript{2+} uniport provides unique possibilities for the elucidation of the mitochondrial Ca\textsuperscript{2+} uniport mechanisms, and to explore the physiological and pathological roles of this process.

**METHODS**

**Animals.** Mice breeding and experiments were performed according the standards established by the Austrian Federal Ministry of Education, Science and Culture, Division of Genetic Engineering and Animal Experiments (Vienna, Austria).

**Cell culture and transfection.** The human umbilical vein endothelial cell line EA.hy926 (ref.16; passage 45–85), freshly isolated endothelial cells from the human umbilical vein (passage 2–4), CHO-K1 and HeLa cells were used in this study. Cells were isolated, cultured and transfected as previously described\textsuperscript{22}.

**Plasmid construction for UCP overexpression.** cDNAs encoding full-length human UCP2 and UCP3 were amplified by RT-PCR from total RNA from EA.hy926 cells\textsuperscript{23}. The forward and reverse primers were: 5′-GGGTTACCCGAGAAGTACG GCATCATGG-3′ and 5′-CCGCTCTGAGTAGTAGAAGACAGGAGGTG-3′ for UCP2 (positions 365 and 3535, respectively; GenBank accession no. NM_003355); 5′-GGGTTACCCGAGGCTTCCAGGACTAT-3′ and 5′-CCGCTCTCAGCTTGTCCTGTTTGAAACGG-3′ for UCP3 (positions 186 and 1153, respectively; GenBank accession no. NM_003356). Subclones were used into the KpnI–XhoI sites of the EF-1α multiple cloning site of the mammalian expression vector pBuCE4.1 (Invitrogen, Carlsbad, CA). For cytosolic free Ca\textsuperscript{2+} measurements, cells were transiently transfected with pBuCE4.1 containing either UCP2 or UCP3 cDNA in the EF-1α-controlled multiple cloning site and mitochondria-targeted DsRed in the CMV-controlled multiple cloning site. For mitochondrial free Ca\textsuperscript{2+} measurements, mitochondria-targeted ratiometric pericam\textsuperscript{17} was subcloned into the HindIII–EcoRI site of the CMV–MCS of UCP-containing vector. Targeting was analysed using UCP–sapphire fusion proteins in pBuCE4.1.

**Mutagenesis of UCPs.** Amino acids 160–169 of human UCP2 and amino acids 163–172 of human UCP3 were replaced by glycines by PCR cloning. cDNAs of mutated UCP genes were cloned into the pBuCE4.1. As for the wild-type UCPs, sapphire fusion proteins of all mutated UCPs were generated.

**Plasmid construction for UCP2 mRNA interference.** pBu6 plasmid was constructed by replacing the CMV promoter of pBuCE4.1 by the U6 promoter of pSuppressor (Imgenex, Sorrento Valley, CA). To generate pBu6–UCP siRNA target sequence, oligonucleotides were designed that contained a unique 19-nucleotide double-stranded human UCP target sequence, present as an inverted repeat, separated by a loop of a 9-nucleotide spacer, and flanked by a 5-T RNA-polymerase III terminator sequence at the 3′-end. The matching strands were annealed to double-stranded DNA and inserted behind the U6 promoter of pBu6. The oligonucleotides against UCP2 mRNA had the following sequence: 5′-TCGAGGCGTGTATGATCTCTGGATACAGAGTTCCCACTTTTTTTC-3′ and 5′-CTAGGAAAAAGGCCGTATGATCTCTGGATACAGAGTTCCCACTTTTTTTC-3′. The 19-nucleotide sequence corresponds to position 672–690 of UCP2 (GenBank accession no. NM_003355). Oligonucleotides that did not reduce the UCP2 protein levels, and did not affect mitochondrial and/or cytosolic Ca\textsuperscript{2+} signalling in response to cell stimulation, were used as non-functional siRNA controls in all experiments in which siRNAs against either UCP2 and/or UCP3 were used. The sequences for these oligonucleotides were: 5′-TCGAGGCGTGTATGATCTCTGGATACAGAGTTCCCACTTTTTTTC-3′ and 5′-CTAGGAAAAAGGCCGTATGATCTCTGGATACAGAGTTCCCACTTTTTTTC-3′. The 19-nucleotide sequence corresponds to position 672–970 of UCP2 (GenBank accession no. NM_003355). Final plasmids containing the inserts were verified by restriction endonuclease digestion and automated fluorescent DNA sequencing. For silencing UCP3 expression, double-stranded RNAs from Quigen (Hilden, Germany) were used.

**Isolation of mitochondria from mouse liver and Saccharomyces cerevisiae.** Mitochondria from liver of wild-type and Ucp2\textsuperscript{–/–} mice, and yeast, were isolated according to Storrie and Madden\textsuperscript{25} and Daum et al\textsuperscript{22}, respectively (see Supplementary Information, Methods).

**Ca\textsuperscript{2+} measurements.** Free cytosolic Ca\textsuperscript{2+} concentration was monitored ratiometrically as ratio signals at 340 nm and 380 nm excitation and 510 nm emission using fura-2 (ref. 23).

To monitor free mitochondrial Ca\textsuperscript{2+} concentration, cells were cotransfected with mitochondria-targeted ratiometric-pericam\textsuperscript{17} and measured using a fluorescence microscope (Eclipse TE300, Nikon, Vienna, Austria) as previously used for mitochondrial Ca\textsuperscript{2+} measurements in cells expressing either UCP2 or UCP3. For the first time, two proteins with known sequence are characterized as fundamental for mitochondrial Ca\textsuperscript{2+} uniport. Although, it remains unclear whether or not UCP2 and UCP3 represent the channel proteins responsible for mitochondrial Ca\textsuperscript{2+} sequestration, the characterization of these proteins as fundamental contributors to mitochondrial Ca\textsuperscript{2+} uniport provides unique possibilities for the elucidation of the mitochondrial Ca\textsuperscript{2+} uniport mechanisms, and to explore the physiological and pathological roles of this process.
von der mitocondrialen Calciumaufnahme abhängig, so dass die Regulation der ATP-Synthese durch Calcium ein wesentliches Element des mitochondrialen Regulationsmechanismus ist. Die Regulation der ATP-Synthese durch Calcium wirkt sich auf die Funktion der mitochondriellen Membranpotenzial und der Mitochondrienphysiologie aus.

Zusammenfassung

Die Untersuchung der Rolle des mitochondrialen Calcium-Transporters in der Regulation der ATP-Synthese durch Calcium zeigte, dass die Regulation der ATP-Synthese durch Calcium ein wichtiger Aspekt der mitochondrialen Funktion ist. Die Regulation der ATP-Synthese durch Calcium wirkt sich auf die Funktion der mitochondriellen Membranpotenzial und der Mitochondrienphysiologie aus.

Literatur


Suppl. Fig. 1a

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Suppl. Fig. 1b

Control

Suppl. Fig. 1c

Suppl. Fig. 1d

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Suppl. Fig. 1e

Graph showing ATP levels over time with different treatments.

Suppl. Fig. 1f

Bar graph illustrating focal contacts of the ER with mitochondria and mitochondria with the ER.

Suppl. Fig. 1g

Images and graphs depicting number of single mitochondria per cell with and without FCCP treatment.

III

Images and graphs showing changes in mitochondrial area and volume with different treatments.
Figure S1. Expression profiling, functional testing and comparison with UCP1.

1a. Profile of UCP expression in various freshly isolated human endothelial cells. Overview of the detection of UCP mRNAs in various cell types (left upper panel). For mRNA detection of the UCP family members, RT-PCRs were performed according to standard procedures using the detection primers given in the left lower panel. The right panels show the agarose gels of the respective.

1b. Overexpression of either UCP2 or UCP3 did not affect the architectural organisation of mitochondria in EA.hy926 cells. For the overexpression of UCP2 or UCP3 in double labelling experiments, cells were transiently transfected with 2 µg DNA/ml of YC4er (ER in green) in pcDNA3 and mitochondria-targeted DsRed (mitochondria in red) (Control, left panels) or together with either UCP2 (middle panels) or UCP3 (right panels) that were cloned into the two multicloning sites of pBudCE4.1, 48 h after transfection, z-scans were performed using an array confocal laser scanning microscope (ACLSM) and 3D-reconstruction and focal contact analysis were performed (see Methods section). Upper panels show representative overlay images of ER and mitochondria, middle panels show the mitochondria with focal contact areas with the ER in yellow, and lower panels show the ER with focal contact areas with mitochondria in yellow. The statistical evaluation of the focal contacts between the mitochondria and the ER are presented in Suppl. Fig. 1e.

1c-I: Expression of UCP2 or UCP3 did not affect the Ca2+ dynamic of the Ca2+-sensitive wavelengths (F433) of mitochondria-targeted pericam. Basal, maximal and minimum F433 values were measured in high KCl buffer (in mM: 135 KCl, 10 NaCl, 1 MgCl2, 20 Hepes; pH 7.7) under nominal Ca2+ free (1 mM EGTA; minimum) and saturating high Ca2+ conditions (10 mM; maximum) in the presence of 5 µM ionomycin, 5 µM digitonin, 4 µM FCCP and 2 µM oligomycin.

1c-II: Mitochondrial matrix pH remained unchanged by overexpression of UCP2 or UCP3. To estimate whether or not overexpression of UCP2 or UCP3 affects basal pH in the mitochondria human endothelial cells were transiently transfected with mitochondria-targeted ratiometric pericam (Control, n=14; or cotransfected with mitochondria-targeted pericam and UCP2 (n=11) or UCP3 (n=14). Basal mitochondrial pH was determined using the pH-sensitive wavelength of mitochondria-targeted ratiometric pericam (i.e. 485 nm excitation). Mitochondria were acidified with 2 µM FCCP in the presence of 5 µM ionomycin, 5 µM digitonin, and 2 µM oligomycin.

1d. Overexpression of either UCP2 or UCP3 did not affect the membrane potential of the inner mitochondrial membrane (ψmito) in EA.hy926 cells. As the fluorescence of mitochondrionally accumulated JC-1 is proportional to the mitochondrial membrane potential2this potentiometric dye was used to assess ψmito. In endothelial cells expressing either mitochondrial-targeted DsRed alone or together with UCP2 or UCP3 the intracellular distribution of JC-1 was assessed by confocal microscopy (panel I, ACLSM, see Methods section). Addition of 40 nM JC-1 to the perfusion medium caused a rapid accumulation of this dye within mitochondria, whereas the fluorescence of JC-1 only slowly increased to a much lesser extent in extra-mitochondrial areas of the cell. Upon addition of 2 µM of the chemical uncoupler FCCP after JC-1 loading a fast redistribution of the potentiometric dye from mitochondria to extra-mitochondrial areas could be observed (panel II). Consequently, the ratio between the average intensity of the JC-1 fluorescence within mitochondria (Fmito) and of a extra-mitochondrial area (Fcyto) was used as an indicator of ψmito, which is independent of dye-loading, light scattering and other optical factors. Neither overexpression of UCP2 nor that of UCP3 affected the intracellular distribution of JC-1 under basal conditions (Control n=12, UCP2 n=9, UCP3 n=11, -FCCP). In line with this finding, addition of FCCP resulted in an equal decline of the Fmito/Fcyto ratio regardless of the UCP2/3 expression levels (Control n=5, UCP2 n=4, UCP3 n=7, +FCCP) (panel III). These measurements clearly indicate that under basal conditions ψmito is not affected by UCP2 or UCP3 overexpression in the endothelial cells used in this study. In an analogous manner Fmito/Fcyto ratio values were obtained in the presence of histamine (100 µM) and histamine plus FCCP, indicating that under condition of cell stimulation with an IP3 generating agonist, ψmito is also not modified by an overexpression of either UCP2 or UCP3 (numbers of experiments: Control, n=7; UCP2, n=5; UCP3, n=5; +Histamine / FCCP, n=5) (panel IV).

1e. Basal ATP concentration in endothelial cells was not affected by overexpressing UCP2 or UCP3. A, Endothelial cells were transiently transfected with cytosolic luciferase together with either mitochondria-targeted DsRed alone (Control, n=7) or with UCP2 (n=7) or UCP3 (n=7). After the addition of 40 µM luciferin, bioluminescence that reflects cytosolic ATP concentration was monitored in cell monolayers.

1f. Overexpression of either UCP2 or UCP3 did not affect the focal contacts of mitochondria with the ER in EA.hy926 cells. Overexpression of neither UCP2 nor UCP3 changed the percentage of focal contacts of the ER with mitochondria and the fraction of mitochondria that co-localised with the ER (Supp. Fig. 1b; Control: n=22; UCP2: n=6; UCP3: n=22; 0.31 < p < 0.65). The respective volumes of co-localisation, where due to the optical resolution of the system an explicitly discrimination of the two organelles could not be achieved, are represented in yellow combined with mitochondria (red) or ER (green) in the middle and right panels, respectively. These focal contacts between ER and mitochondria were defined as the percentage values of voxels that contain both fluorescent proteins, which were obtained from the co-localisation analysis module in Imaris 3.3. The surface area and the volume of mitochondria per cell were not affected by overexpression of either UCP2 or UCP3 (panel I; Control: n =22; UCP2: n=6; UCP3: n=22; 0.09 < p > 0.20). The interconnected, tubular appearance of mitochondria within individual endothelial cells and the number of single mitochondria per cell were also not altered by overexpression of these UCP orthologs. In contrast to the overexpression of UCP2 and UCP3, the chemical uncoupler FCCP (2µM) initiated fragmentation of mitochondria within 5 minutes (panel II). FCCP treatment significantly increased the number of single mitochondria per cell from 83±15 to 159±15 in control cells (n = 10) and from 90±16 to 143±21 in cells overexpressing UCP3 (n=8) within 5 minutes (panel III). “n” values indicate the number of cells from at least three different experiments.

1g. Basal ER Ca2+ content and histamine-induced Ca2+ release remained unchanged by overexpression of UCP2 or UCP3. A panel I: Endothelial cells were transiently transfected with mitochondria-targeted DsRed alone (Control, n=11) or together with UCP2 (n=12) or UCP3 (n=12), loaded with fura-2/3/am (see Methods) and cytosolic Ca2+ elevations in response to 100 µM histamine in the nominal absence of extracellular free Ca2+ (i.e. no Ca2+ added plus 1 mM EGTA) were fluorometrically recorded in single cells. Panel II and III: Endothelial cells were transfected with D1ER, an ER targeted FRET based Ca2+ sensor. The basal F433/F480 ratio of this ratiometric ER Ca2+ sensor was not affected upon overexpression of either UCP2 or UCP3 (Control: n=6; UCP2: n=5; UCP3: n=6) indicating that the global ER Ca2+ content under basal conditions was not altered by an overexpression of these UCP-orthologs. In line with these results histamine induced ER Ca2+ depletion was identical in control cells (n=6), UCP2 overexpressing cells (n=5) and UCP3 overexpressing cells (n=6) in the presence as well as in the absence of extracellular Ca2+. “n” values indicate the number of cells from at least three different experiments.
1h. Cytoplasmic Ca\textsuperscript{2+} elevation in response to histamine was not affected in UCP overexpressing cells. Endothelial cells were transiently transfected with either mitochondria-targeted DsRed alone (Control, n=17), or together with UCP2 (n=21) or UCP3 (n=16), loaded with fura-2/am (see Methods) and cytosolic Ca\textsuperscript{2+} elevations in response to 100 µM histamine in the presence of 2 mM free extracellular Ca\textsuperscript{2+} were fluorometrically recorded in single cells. "n" values indicate the number of cells from at least ten different experiments.

1i. Increased mitochondrial Ca\textsuperscript{2+} sequestration in UCP overexpressing cells was independent from the source of Ca\textsuperscript{2+}. Endothelial cells were transiently transfected with mitochondria-targeted ratiometric pericam alone (Control, n=9) or together with UCP2 (n=9) or UCP3 (n=9). Mitochondrial Ca\textsuperscript{2+} concentration was recorded in single cells as described under Methods. As indicated, cells were stimulated with 100 µM histamine in the absence of extracellular Ca\textsuperscript{2+} followed by readdition of 2 mM extracellular Ca\textsuperscript{2+}. *P>0.05 vs control. "n" values indicate the number of different experiments.

1j. As in endothelial cells, overexpression of UCP2 or UCP3 yielded increased mitochondrial Ca\textsuperscript{2+} sequestration in HeLa and CHO-K1 cells. HeLa (left panel) and CHO-K1 (right panel) cells were transiently transfected with mitochondria-targeted ratiometric pericam alone (Control, HeLa: n=11; CHO-K1: n=13) or together with UCP2 (HeLa: n=9; CHO-K1: n=10) or UCP3 (HeLa: n=10; CHO-K1: n=11). As indicated, HeLa and CHO-K1 cells were stimulated with 100 µM histamine or 10 µM ATP, respectively. *P>0.05 vs control. "n" values indicate the number of cells from at least six different experiments.

1k. Expression of UCP1 did not affect mitochondrial Ca\textsuperscript{2+} sequestration upon stimulation with an agonist. EA.hy926 cells were transiently transfected with mitochondria-targeted ratiometric pericam alone (Control, n=13) or together with UCP1 (n=15). As indicated, cells were stimulated with 100 µM histamine. Mitochondrial Ca\textsuperscript{2+} concentration was recorded in single cells as described under Methods. "n" values indicate the number of cells from at least six different experiments.

1l. Efficiency of FCCP to depolarize mitochondria in the endothelial cells. In order to assess whether or not 2 µM FCCP is enough to completely degrade ψ\textsubscript{mito} within 3 minutes in the respective cell model (see mitochondrial Ca\textsuperscript{2+} uptake under depolarizing conditions Fig. 1e) different concentrations of FCCP from 0.1 to 10 µM were used and JC-1 redistribution was monitored as described in Suppl. Fig. 1d. Δψ\textsubscript{mito} did not significantly change upon increasing the FCCP concentration from 0.1 to 10 µM (n=3 for each concentration) indicating that within 3 minutes the maximal effect of FCCP on ψ\textsubscript{mito} was achieved with the usage of 2 µM of this chemical uncoupler.
Supl. Fig. 2

**a**

System 1: In vivo expression of shRNA for UCP2 silencing

**b**

System 2: Direct delivery of double stranded RNA for UCP3 silencing

**Figure S2.** Knock-down of UCP2 or UCP3. 2a. Design of siRNA against UCP2 and UCP3. Upper panel: In the commercially available vector pBudCE4.1 the existing CMV promotor was exchanged for the U6 promotor using Sal I and BamHI I. Subsequently, a functional or non-functional siRNA hairpin-forming sequence (upper box) against UCP2 was introduced into the Spe I / Sal I cloning site behind the U6 promotor. Additionally, purchased siRNA duplexes for UCP3 silencing (Qiagen, Hilden, Germany, Cat No.: SI00051170, functional siRNA against UCP3; Cat No.: SI0051177, non-functioning siRNA-control) were co-transfected as indicated (lower box).

2b. The siRNAs against UCP2 and UCP3 did not affect histamine-induced cytosolic Ca^{2+} elevation. EA.hy926 cells were transiently transfected with non-functional siRNA-control (Control, n=9), short hairpin siRNA against UCP2 (n=9) or double stranded RNA for UCP3 silencing (n=9), loaded with fura-2/am (see Methods) and cytosolic Ca^{2+} elevations in response to 100 µM histamine in the presence of 2 mM free extracellular Ca^{2+} were fluorometrically recorded in single cells.
Figure S3. Concerning data in single liver mitochondria from wild type and UCP2<sup>-/-</sup> mice (panels a-c), and single mitochondria freshly isolated from wild type yeast, and UCP2 and UCP3 expressing yeast (panel d). 3a-c: No difference in the membrane potential of suspended liver mitochondria between organelles isolated from wild type and UCP2<sup>-/-</sup> mice was observed. The membrane potential of suspended mitochondria freshly isolated from wild type and UCP2<sup>-/-</sup> mice was measured using JC-1 as in Methods. Panel a shows the statistical summary of all experiments performed (WT, n=6) and UCP2<sup>-/-</sup> (n=6). Panel b and c are representative tracings where suspended mitochondria were added to the JC-1 containing solution as indicated. In order to evaluate the fluorescence at 0 mV membrane potential 4 µM FCCP were added after the JC-1 signal reached a stable plateau phase.

3d. Comparison of mitochondrial Ca<sup>2+</sup> sequestration and its sensitivity to ruthenium red in freshly isolated single mitochondria from wild type yeast, and yeast expressing either UCP2 and UCP3. Ca<sup>2+</sup> uptake in isolated single mitochondria from Saccharomyces cerevisiae diploid, W303 (α/a), transformed with either the plasmid vector pRUCP2 (moderate UCP2 expression) (left panel: UCP2, n=54) or with the pYES2 empty vector as the paired control<sup>3</sup> (left panel: Control, n=52), or with either the plasmid vector pBF354 (UCP3 expression) (right panel: UCP3, n=44) or with pKV49 (empty vector control)<sup>4</sup> (right panel: Control, n=47). After loading with rhod-2 (see Methods below) mitochondrial Ca<sup>2+</sup> sequestration upon addition of 10 µM free Ca<sup>2+</sup> was monitored fluorometrically in the absence or presence of 1 µM ruthenium red (+ RR).
Suppl. Fig. 4

### a

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### b

![Suppl. Fig. 4](image_url)
Suppl. Fig. 4

**Figure S4.** Concerning mutagenesis of the UCP2 and UCP3 (panel a & b) and the effect of UCP2 and UCP3 overexpression on basal (c) and agonist (d)-induced ATP synthesis in the mitochondria. 4a. Comparison of the amino acid sequence homology between UCP1, UCP2 and UCP3. Amino acid sequences of UCP1, UCP2 and UCP3 were aligned according to the predicted transmembrane and intermembranous loop domains and the sequences and homology are presented. The highlighted sequences indicate the area of highest diversity between UCP2/UCP3 and UCP1 in terms of amino acid properties. This area was emphasized to be important for the Ca\(^{2+}\) carrier function of UCP2 and UCP3 and, thus, was selected for mutation. 4b. Like their non-mutant homologues, the mutated UCP2 and UCP3 were localized exclusively in the mitochondria. Fusion constructs of mutated UCP2 (upper panels) or mutated UCP3 (middle panels) with sapphire\(^5\) were expressed and their co-localization with mito-tracker orange\(^6\) was visualized in human endothelial cells. Panels I and IV show the localization of the fusion constructs of mutated UCP2 and mutated UCP3, II and V the localization of mito-tracker orange\(^6\), and III and VI the co-localization in the respective cell. 4c. Consequently to the mitochondrial Ca\(^{2+}\) carrier function of UCP2 and UCP3, agonist-induced mitochondrial ATP production was elevated in UCP overexpressing cells. Endothelial cells were transiently transfected with cytosolic luciferase together with mitochondria-targeted DsRed (Control, n=7), the mitochondria-targeted DsRed and UCP2 (n=7) or the mitochondria-targeted DsRed and UCP3 (n=7). After the addition of 40 µM luciferin bioluminescence was monitored in cell monolayers in the absence or presence of 1mM atractyloside. *P<0.05 vs Control.

**Movie 1:** Overexpression of UCP2 and UCP3 did not affect basal mitochondrial dynamics, fusion and fission. Time scan acquisitions of mitochondrial movement, fusion and fission were performed in cells transiently expressing mitochondria-targeted DsRed (Control) or mitochondria-targeted DsRed and UCP2 (co-transfection) using the ACLSM as described in the Methods section below. At a constant z-plane, images were recorded every 2 s. Exposure time was 1 s. Arrows indicate typical branching of tubular mitochondrial structures. Fluctuations are possibly due to i, small alterations of the z-stage of the motorized microscope, ii, floating cell debris or iii, alterations in the laser power during the experiments.

**Movie 2:** Ca\(^{2+}\) measurements in single mitochondria freshly isolated from yeast Saccharomyces cerevisiae using rhod-2. Changes in rhod-2 fluorescence upon increasing the Ca\(^{2+}\) concentration in the buffer from nominal Ca\(^{2+}\) free buffer to 10 µM Ca\(^{2+}\) were recorded every 5 s using high resolution CCD camera (CoolSnap HQ, Roper Scientific, Visitron Systems, Puchheim, Germany) (for details see Methods below).
Table S1. Non-normalized original data of the mitochondria-targeted ratiometric pericam data provided in the article. The fluorescence of mitochondria-targeted ratiometric pericam at two different excitation wavelengths is mainly Ca²⁺- (F₄₃₃) as well as pH- (F₄₈₅) dependent. Consequently, caution is necessary when presenting ratiometric data under dynamical conditions in which also changes in the pH might occur. Therefore, to demonstrate changes in mitochondrial Ca²⁺ upon cell stimulation data are given as $1 - \frac{F_{433}}{F_0}$. However, since other basal parameters of mitochondria (see Suppl. Fig. 1) did not alter when UCP2 and UCP3 expression was modulated, a statistical evaluation of the original basal data is shown here in order to conclude that there are no differences in basal mitochondrial Ca²⁺ concentration under conditions of various UCP expression. “n” values correspond to the respective figures.
MATERIALS:
If not indicated separately, all cell culture chemicals and pBudCE4.1 were obtained from Invitrogen Corp. (Carlsbad, California, USA) and fetal calf serum (FCS) was from PAA Laboratories (Linz, Austria). Calcium Gree-5N hexapotassium salt, fura-2/am, 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetrachloro-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) and mito-Tracker Orange® were from Molecular Probes Europe (Leiden, Netherlands). ATP, antimycin A, oligomycin, atractyloside, FCCP, histamine, EGTA, luciferin, Ham’s F12 and Dulbecco’s minimum essential medium (DMEM) were from Sigma-Aldrich (Vienna, Austria). CGP 37157 was from Tocris Cookson Ltd. (Northpoint, Avonmouth, Bristol, UK). Endothelial growth medium (EGM) and the bullet-kit were purchased from Cambrex-Clonetics (Verviers, Belgium). Restriction enzymes and T4 DNA ligase were from Promega (Mannheim, Germany), the EndoFree Plasmid Maxi Kit and the double stranded RNA for UCP3 silencing were from Quiagen (Hilden, Germany). All other chemicals were from Roth (Karlsruhe, Germany). The primary antibodies and ECL solution were purchased from Santa Cruz (UCP2, sc-6525; UCP3, sc-7756; Santa Cruz Biotechnology Inc., Szabo-Scandic, Vienna, Austria) and from Abcam® (β-Actin, ab8224; Abcam, Cambridge, UK). As secondary antibodies, peroxidase-conjugated IgGs (rabbit anti-goat IgG, sc-2768, Santa Cruz and rabbit anti-mouse IgG, P 0260, DAKO, Denmark) were used.

METHODS:

Concerning experiments with cultured cells:

ψ_mito Measurement. Cells expressing mitochondria-targeted DsRed were loaded with 40 nM JC-1 on the ACLSM using a gravity based perfusion system (for details see Methods below). JC-1 was continuously present throughout all the experiments in order to counteract possible leakage of the potentiometric dye. Mitochondria-targeted DsRed and JC-1 were excited with the 488 nm Ar-laser line and emission was alternatively monitored every 0.8 s at 570 nm (for mitochondria-targeted DsRed) and 535 nm (for JC-1). The mitochondria-targeted DsRed channel was used to define regions that cover exclusively mitochondria within one given cell.
The average intensity of the JC-1 fluorescence of this regions (F\textsubscript{mito}) was divided by the average intensity of the JC-1 fluorescence of a region that was at least 3 µm away from these organelles (F\textsubscript{cyto}). The F\textsubscript{mito}/F\textsubscript{cyto}-ratio was used to assess changes in ψ\textsubscript{mito}.

**Confocal imaging and 3D rendering.** Z-scans were performed in cells that were co-transfected with YC4er and mitochondria-targeted DsRed using a Nipkow-disk-based array confocal laser scanning microscope (ACLSM). The ACLSM was built on a Zeiss Axiovert 200M (Zeiss Microsystems, Jena, Germany) equipped with VoxCell Scan® (VisiTech, Sunderland, UK), a 150 mW Ar laser (Laser Physics; West Jordan, UT, USA) and controlled by Metamorph 6.2r6 (Universal Imaging, Visitron Systems, Puchheim, Germany). The green fluorescent probe targeted to the ER (i.e. YC4er) and the red fluorescence within mitochondria (i.e. mitochondria-targeted DsRed) were alternatively imaged with a 100 x objective (α Plan-Fluar 100 x / 1.45 oil objective, Zeiss Microsystems, Jena, Germany) using the 488 nm and 514 nm Ar-laser lines for illumination. Z-interval between the planes was 0.1 µm. The emitted light was filtered at 535 nm and 570 nm using two different emission filters (535/30 and E570LPv2, Chroma Technology Corp., Rockingham, VT, USA), which were mounted in a computer-controlled fast filter wheel (Ludl, Electronic Products, Hawthorne, NY, USA). The ER and mitochondria z-stacks were deconvoluted using the iterative quick maximum likelihood estimation algorithm (QMLE) of Huygens 2.4.1p3 (SVI, Hilversum, Netherlands). Subsequently combined 3-D rendering of the organelles, co-localisation analysis, and mitochondrial surface and volume calculations were performed with Imaris 3.3 software (Bitplane AG, Zürich, Switzerland). The intensity threshold values for 3D image restorations and co-localisation computations did not significantly differ within all the samples analysed and were determined over a range that completely eliminated background fluorescence but preserved organelle structures. Focal contacts between ER and mitochondria were defined as the percentage values of voxels that contain both fluorescent proteins, which were obtained from the co-localisation analysis module in Imaris 3.3. Notably, due to the limitation in regard to the optical resolution the percentage values for focal contacts between the ER and mitochondria and vice versa are probably overestimated.
**Luminescent ATP assays.** Bioluminescent measurements of cellular ATP levels were performed according to Jouaville et al. All luminescence experiments were performed in a GLOMAX luminometer with 10-seconds integration time (Turner Biosystems, Inc., CA, USA). For intracellular ATP measurements, EA.hy926 cells were seeded on 3cm plates one day before transfection. Cells at app. 80 % confluency were co-transfected with cytosolic luciferase and mitochondria-targeted DsRed for control experiments or co-transfected with cytosolic luciferase and UCP2 or UCP3 mitochondria-targeted DsRed for UCP-overexpressor experiments. 48 h after transfection cells were washed twice with Hepes-buffered Ca$^{2+}$ solution (EB) and measurement of luminescence was started after adding 40 µM luciferin. 100 µM histamine was added 30 min past start. As indicated, 1mM atractyloside was applied to the cells.

**Concerning the experiments in single and suspended liver mitochondria freshly isolated from wild type and knockout mice:**

**Isolation of mitochondria from mouse liver.** Liver mitochondria from wild-type (WT) and UCP2 knock-out (UCP2$^{-/-}$) mice were isolated by differential centrifugation as described by Storrie and Madden followed by a sucrose-gradient centrifugation to further purify the mitochondrial fraction. Shortly, the fresh tissue sample was washed twice with 2 volumes of extraction buffer EB (in mM: 10 Hepes, 250 sucrose and 1 EGTA, pH 7.5), cut into small slices and homogenized in 10 ml EB per g tissue containing 2 mg/ml BSA in a Potter homogenizer with a Teflon pestle (B. Braun, Melsungen, Germany). To separate the mitochondria from other organelles and cell debris the homogenate was centrifuged at different speeds. The obtained raw mitochondrial pellets were then placed on top of a 2-step sucrose gradient consisting of a 1.5 M sucrose and a 1.0 M sucrose phase and centrifuged 25 min at 45000 g. Purified mitochondria were collected from the interphase, pelleted and resuspended in storage buffer SB (in mM: 10 Hepes, 250 sucrose, 1 ATP, 0.08 ADP, 5 succinate, 2 KH$_2$PO$_4$, 1 DTT, pH 7.4). All steps were carried out at 0-4°C.

**Measurement of the mitochondrial membrane potential.** The membrane potential of liver mitochondria isolated from wild-type and UCP2$^{-/-}$ mice was measured on a Hitachi F-4500
fluorescence spectrometer at room temperature using JC-1 (Molecular Probes). 2 µL of JC-1 (0.2 mg/ml) were added to 2 ml of KCl-buffer (see above). A dual-wavelength program allowed to monitor a time-dependent kinetics of both, the green fluorescent monomers and the red fluorescent J-aggregates, with excitation/emission wavelength of 490nm/530nm and 490nm/590nm, respectively. The ratios between red fluorescence vs. green fluorescence \((F_{590}/F_{530})\) reflect the mitochondrial loading of JC-1. Following an initial period of incubation, a suspension of isolated mitochondria (0.12 mg of protein) were added to the stirred buffer and the uptake of JC-1 into the mitochondria was monitored in 2 second intervals for up to 20 minutes, since both wavelengths reached a plateau after approx. 15 minutes. At the end of each experiment 4µM FCCP was added, leading to a depolarisation of the mitochondria and a drastic decrease of red fluorescence of JC-1 due to equalization of the JC-1 fluorescence inside and outside the mitochondrial matrix.

Concerning the experiments in single mitochondria freshly isolated from yeast

*Saccharomyces cerevisiae* expressing either UCP2 or UCP3:

Expression of human UCP2 and UCP3 at physiological levels in the yeast *Saccharomyces cerevisiae*. For the expression of human UCP2 and UCP3 in yeast we used cells of the *Saccharomyces cerevisiae* diploid, W303 (a/α), transformed with either the plasmid vector pRUCP2 (moderate UCP2 expression) or with the pYes2 empty vector as the paired control, or with either the plasmid vector pBF354 (UCP3 expression) or with pKV49 (empty vector control). Procedures of UCP2 and UCP3 expressions were performed according the published standard protocols. According to the provider, these procedure yields expression of UCP2 and UCP3 on a similar level in yeast mitochondria as found in mammalians.

Isolation of mitochondria from *Saccharomyces cerevisiae*. Mitochondria from *Saccharomyces cerevisiae* were isolated as described by Daum et al. Cells were harvested at an \(D_{600}\) of approx. 2 by centrifugation at 3000 g for 5 min at room temperature, washed once with distilled water, suspended to 0.5 g wet weight/ml in 0.1 M TrisSO₄, pH 9.4, 10 mM DTT, and incubated for 10 min at 30°C. Subsequently, the cells were re-centrifuged and
washed once, and suspended in 1.2 M sorbitol, 20 mM KH$_2$PO$_4$, pH 7.4, to give 0.15 g cell wet weight/ml. Zymolyase 20T (2 mg/g cell wet weight) was added and the suspension was incubated at 30 °C for 45-60 min. Spheroplasts were pelleted at room temperature, washed with 1.2 M sorbitol and resuspended in ice cold buffer containing 0.6 M mannitol, 10 mM TrisCl, pH 7.4 and 1mM PMSF to a concentration of 0.15 g spheroplasts/ml. All subsequent operations were carried out at 4 °C. Spheroplasts were homogenized by 15 strokes in a Dounce homogenizer. The homogenate was centrifuged for 5 min at 3500 g, the supernatant was saved and the pellet was rehomogenized two times as before. The combined supernatants were centrifuged 10 min at 12000 g to sediment the crude mitochondrial fraction. The pellet was carefully resuspended in homogenisation buffer and centrifuged for 5 min at 3500 g to remove residual cell debris. The supernatant was then centrifuged for 10 min at 12000 g and the final mitochondrial pellet was washed once with PMSF free homogenisation buffer and resuspended in storage buffer SB at a concentration of 8 mg/ml mitochondrial protein.

**Loading of yeast mitochondria with rhod-2/am.** The mitochondrial suspension with a concentration of approx. 8mg/ml of mitochondrial protein was loaded with 2 µM rhod-2/am for 60 min in the dark at room temperature under continuous stirring. The mitochondria were then washed 2 x with 2 volumes and 1 x with 1 volume SB. Finally, mitochondria were resuspended in assay buffer AB (in mM: 20 Hepes, 130 KCl, 1 MgCl$_2$, 0.5 KH$_2$PO$_4$, 10 succinate, 2 malate, 0.5 EGTA, 0.6 ATP, 0.05 ADP, pH 7.2) at a final concentration of 8 mg/ml mitochondrial protein.

**Fluorescence measurements of single mitochondria isolated from yeast.** Measurements of yeast mitochondria Ca$^{2+}$ signal were performed using rhod-2. Rhod-2-containing organelles were excited at 546 nm (D546/10) and emitted light was collected at >590 nm (e590v2lp; dichroic: 565dclp); 2 s exposure time) upon changing the perfusion solution from Ca$^{2+}$ free buffer (AB) to a 10 µM Ca$^{2+}$ containing buffer (see Movie 2). Experiments were performed using an inverted fluorescence microscope (Zeiss Axiovert 200M) equipped with a 100x oil immersion objective (NA=1.45, Zeiss, Vienna, Austria).
REFERENCES to the supplementary material:


7. Malli, R. et al. Sustained Ca\textsuperscript{2+} transfer across mitochondria is essential for mitochondrial Ca\textsuperscript{2+} buffering, store-operated Ca\textsuperscript{2+} entry, and Ca\textsuperscript{2+} store refilling. J Biol Chem 278, 44769-44779 (2003).


