SCaMC-1 promotes cancer cell survival by desensitizing mitochondrial permeability transition via ATP/ADP-mediated matrix Ca^{2+} buffering

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Ca^{2+} -mediated mitochondrial permeability transition (mPT) is the final common pathway of stress-induced cell death in many major pathologies, but its regulation in intact cells is poorly understood. Here we report that the mitochondrial carrier SCaMC-1/SLC25A24 mediates $\text{ATP-Mg}^{2+}/\text{Pi}^{2-}$ and/or $\text{HADP}^{2-}/\text{Pi}^{2-}$ uptake into the mitochondria after an increase in cytosolic $[\text{Ca}^{2+}]$. ATP and ADP contribute to Ca^{2+} buffering in the mitochondrial matrix, resulting in desensitization of the mPT. Comprehensive gene expression analysis showed that SCaMC-1 overexpression is a general feature of transformed and cancer cells. Knockdown of the transporter led to vast reduction of mitochondrial Ca^{2+} buffering capacity and sensitized cells to mPT-mediated necrotic death triggered by oxidative stress and Ca^{2+} overload. These findings revealed that SCaMC-1 exerts a negative feedback control between cellular Ca^{2+} overload and mPT-dependent cell death, suggesting that the carrier might represent a novel target for cancer therapy.

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ATP generation by aerobic glycolysis is indispensable for survival and proliferation of many tumour cell types, associated with resistance to stress-induced apoptotic and necrotic death.^{1,2} In many cell death models, mitochondrial permeability transition (mPT) has a central role in permeabilizing the inner mitochondrial membrane (IMM), leading to necrotic cell death.³ The mPT is triggered by the formation of a large (cut-off ~ 1.5 kDa), non-specific pore (mPTP) in the IMM under conditions of Ca^{2+} overload and/or oxidative stress. mPTP opening dissipates the mitochondrial membrane potential ($\Delta\psi$) and can be associated with mitochondrial swelling, particularly under *in vitro* conditions.⁴ Although the molecular composition of the mPTP is still debated, pharmacological and genetic evidence strongly supports its implication in cell death in a wide range of pathologies.⁵ Accordingly, resistance of tumour cells to death could be explained by decreased probability of mPTP opening. Indeed, it has been suggested that Bcl-2 overexpression or reduction of Bax/Bak expression can mediate protective effect by diminishing mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{m}}$) load from the endoplasmic reticulum (ER) Ca^{2+} store.⁶ In addition, direct modification of putative mPTP components, such as hexokinase-II binding to

the voltage-dependent anion channel (VDAC)⁷ and dephosphorylation of cyclophilin-D (CyP-D),⁸ have also been shown to reduce the sensitivity of mPTP formation to elevations of $[\text{Ca}^{2+}]_{\text{m}}$. However, modification of the mPT is not essential for mediating cancer cell protection,⁹ and no intrinsic mitochondrial factor has been described so far contributing to the development of resistance to mPT in cancer cells.

The net mitochondrial content of adenine nucleotides is central to the regulation of Ca^{2+} -induced mPT in isolated organelles, but the underlying mechanisms remained unclear.¹⁰ The adenine nucleotide translocator (ANT) of the IMM exchanges ATP^{4-} for ADP^{3-} , but does not change the total matrix ATP/ADP/AMP content. The net content of adenine nucleotides may rather be determined by the ATP-Mg/Pi transporter, which mediates a reversible, electroneutral exchange of ATP-Mg^{2-} or HADP^{2-} for HPO_4^{2-} , stimulated by extra-mitochondrial Ca^{2+} .¹¹ Recently, the genes encoding this transporter have been identified. There are four paralogues in mammals, SCaMC-1/SLC25A24, SCaMC-2/SLC25A25, SCaMC-3/SLC25A23 and SCaMC-3-like/SLC25A41.^{12,13} The transporter consists of a C-terminal domain comprising six transmembrane helices homologous

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Abbreviations: $[\text{Ca}^{2+}]_{\text{cyt}}$, cytosolic $[\text{Ca}^{2+}]$; $[\text{Ca}^{2+}]_{\text{m}}$, mitochondrial $[\text{Ca}^{2+}]$; ANT, adenine nucleotide translocator; BKA, bongkrekic acid; CAT, carboxyatractyloside; CRC, Ca^{2+} retention capacity; CsA, cyclosporin-A; CyP-D, cyclophilin-D; cytAEQ, cytosolic aequorin; ER, endoplasmic reticulum; IC, intracellular; IMM, inner mitochondrial membrane; IP_3 , inositol-1,4,5-trisphosphate; MCU, mitochondrial Ca^{2+} uniporter; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; mtAEQmut, low-affinity mitochondrial aequorin; mtAEQwt, mitochondrial aequorin; RR, ruthenium red; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; $\Delta\psi$, mitochondrial membrane potential

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to the mitochondrial carrier proteins,¹⁴ and an N-terminal domain with Ca^{2+} -binding EF hands,¹⁵ which confers Ca^{2+} sensitivity to the carrier.^{11,16} These properties of the carrier prompted us to investigate the role it might have in regulating the Ca^{2+} retention capacity (CRC) of mitochondria and mPTP formation in intact cells.

Here we report that SCaMC-1 is the dominant isoform of the ATP-Mg/Pi carrier in cancer cells and is highly over-expressed in a series of *in vivo* tumours and cell lines. We found that cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$)-mediated uptake of ATP/ADP by SCaMC-1 increases intra-mitochondrial Ca^{2+} buffering, and thus contributes to the resistance to mPT in tumour cells.

Results and Discussion

SCaMC-1 is highly expressed in tumours and cancer cell lines, and mediates Ca^{2+} -dependent ATP-Mg and ADP uptake into mitochondria. First, using western blotting, immunofluorescence and *in silico* approaches we showed that SCaMC-1 is the dominant and highly expressed isoform of ATP-Mg/Pi carrier family in a wide range of tumours, cancer cell lines and highly proliferative immortalized cells (Figures 1a–c and Supplementary Figure S1). Therefore, in order to study the role of SCaMC-1 in mitochondrial adenine nucleotide transport and cancer cell fate, we generated stable SCaMC-1-knockdown (SCaMC-1-KD) cell lines by using COS-7 and 143B parental lines (Figures 1d–g). In the SCaMC-1-KD COS-7 and 143B clones, expression was reduced to $29.6 \pm 3.8\%$ and $41.7 \pm 5.0\%$, respectively (Figure 1f).

Next, in order to evaluate mitochondrial adenine nucleotide transport in the parental and SCaMC-1-KD clones, cells were transiently or stably transfected with mitochondrial matrix-targeted luciferase to measure mitochondrial ATP levels in digitonin-permeabilized cells or isolated mitochondria.¹⁷ After cell permeabilization in an intracellular (IC) buffer supplemented with luciferin, the added ATP was imported into the mitochondria (Figure 2). Under Ca^{2+} -free conditions uptake of ATP was completely inhibited by $10 \mu\text{M}$ carboxyatractyloside (CAT), indicating that it was mediated entirely by ATP/ADP exchange through ANT. Ca^{2+} -independent ATP import was identical in parental and SCaMC-1-KD cells (Figure 2a). However, in the presence of $100 \mu\text{M}$ Ca^{2+} , ATP was rapidly imported into the mitochondrial matrix even in the presence of CAT (Figure 2b). The transport was activated by extra-mitochondrial Ca^{2+} , as 200 nM ruthenium red (RR) was present in all the experiments to prevent Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU). Under these conditions, ADP uptake was also observed, as measured after its conversion to ATP owing to oxidative phosphorylation in the matrix (Figure 2c and Supplementary Figure S2A).¹⁶ Chelation of Mg^{2+} (with 1 mM EDTA) reduced the ATP uptake rate (not shown). The dependence of CAT-insensitive ATP transport on extra-mitochondrial $[\text{Ca}^{2+}]$ was determined on isolated mitochondria by using a mitochondrial luciferase-expressing COS-7 cell line, showing half-maximal activation at $[\text{Ca}^{2+}] = 12.7 \pm 5.3 \mu\text{M}$ (Figures 2d and f). Pi addition reversed the activity of the transporter (Figure 2e), with a

similar Ca^{2+} dependence. These results confirmed that these cells express a bona fide Ca^{2+} -dependent ATP-Mg/Pi carrier, mediating the exchange of ATP-Mg and free ADP to Pi.¹⁸

Importantly, the CAT-insensitive/ Ca^{2+} -dependent ATP-Mg/ADP transport was strongly reduced in SCaMC-1-KD cells (Figures 2b and c; ATP: to $56 \pm 8\%$ and ADP: to $55 \pm 5\%$ of the control rate) to an extent similar to the reduction of SCaMC-1 expression, demonstrating that SCaMC-1 mediates ATP-Mg/Pi transporter activity facilitated by extra-mitochondrial $[\text{Ca}^{2+}]$.

SCaMC-1 protects cancer cells from oxidative stress-induced death. High expression levels of SCaMC-1 in tumours and cancer cell lines suggested that the carrier confers selective advantage to proliferation either by altering cellular metabolism or by mediating protection against cell death. We observed no effect on cellular energy metabolism and proliferation (see Supplementary Figure S3). Thus next, in order to test whether SCaMC-1 has a role in modulating cell death pathways, control and SCaMC-1-KD cells were exposed to (i) oxidative stress by H_2O_2 or menadione treatment, leading to mitochondrial Ca^{2+} overload, mPT and necrosis; or (ii) staurosporine, triggering the intrinsic pathway of apoptosis. As shown in Figures 3a–c, reduced expression of SCaMC-1 rendered cells more susceptible to oxidative stress-induced cell death (Figures 3a and b), whereas it had no effect on apoptosis induced by staurosporine (Figures 3c and Supplementary Figure S4A). These data indicated that high expression levels of SCaMC-1 confer resistance specifically to mPT-dependent cell death.

In order to directly demonstrate the effect of SCaMC-1 silencing on mPT in intact cells, we studied Ca^{2+} - and reactive oxygen species (ROS)-mediated mitochondrial depolarization by confocal microscopy, and a previously established model of phototoxicity.¹⁹ After tetramethylrhodamine methyl ester (TMRM) loading, cells were illuminated with high laser power, which leads to local generation of ROS in the mitochondria. ROS-mediated Ca^{2+} release from the ER results in mitochondrial Ca^{2+} overload and mPT, visualized by loss of TMRM. As shown in Figure 3d, illumination of parental COS-7 cells had no effect on the mitochondrial membrane potential ($\Delta\Psi_m$), whereas it led to complete loss of $\Delta\Psi_m$ in SCaMC-1-KD cells. The effect was Ca^{2+} -dependent, as it was completely prevented in Ca^{2+} -free medium (in the presence of $100 \mu\text{M}$ EGTA) and by depleting the internal Ca^{2+} stores using thapsigargin ($1 \mu\text{M}$). Moreover, addition of cyclosporin-A (CsA) and bongrekic acid (BKA), inhibitors of mPT,²⁰ prevented mitochondrial depolarization in SCaMC-1-KD cells, confirming that it was indeed due to mPT.

Finally, to test whether the high level of SCaMC-1 expression is indeed responsible for protection against mPT-dependent cell death, we (i) rescued the expression of SCaMC-1 in SCaMC-1-KD 143B cells, using a mutant cDNA containing synonymous mutations (SCaMC-1*), and (ii) overexpressed the protein in liver clone-9 cells with low levels of endogenous SCaMC-1 (Supplementary Figure S1). Re-expression of SCaMC-1 in SCaMC-1-KD 143B cells (Figure 3e), as well as its overexpression in both a transient (Figure 3e) or a stable (Figure 3f) manner in clone-9 cells rendered cells more resistant to H_2O_2 - or C_2 ceramide-induced

cell death.²¹ These results confirmed that SCaMC-1 mediates protection against mPT-induced cell death.

SCaMC-1-mediated mitochondrial Ca^{2+} uptake controls ATP/ADP buffering capacity. Elevation of

mitochondrial matrix free $[\text{Ca}^{2+}]$ is the most potent inducer of mPT. To determine the effect of SCaMC-1 on cellular and mitochondrial Ca^{2+} handling in intact cells, we measured agonist-evoked cytosolic and mitochondrial Ca^{2+} transients in cells transiently transfected with a cytosolic aequorin

Figure 2 Ca^{2+} -dependent/CAT-insensitive adenine nucleotide uptake through SCaMC-1. (a–c) 143B cells transiently expressing mitochondrial luciferase¹⁷ were permeabilized and 100 μM luciferin was added to report mitochondrial ATP levels. The black traces indicate control cells, whereas the red traces indicate SCaMC-1-KD cells. (a) ANT activity in the absence of Ca^{2+} : The medium contained 100 μM EGTA and 1 mM ATP was added when indicated. The traces marked by arrows were obtained from cells treated with 10 μM CAT. (b and c) SCaMC-1 activity: The medium contained 100 μM Ca^{2+} and 10 μM CAT, and 1 mM ATP (b) or 2 mM ADP (c) was added when indicated. The bar charts indicate the transport rate of SCaMC-1-KD (KD) cells as compared with controls (C) (mean $\pm$ S.E.M., $n = 6$). (d–f) To determine the Ca^{2+} sensitivity of SCaMC-1-mediated ATP/ADP transport, mitochondrial ATP changes were measured in isolated mitochondria from COS-7 cells stably expressing mitochondrial luciferase after addition of 100 μM luciferin. (d) The medium contained 10 μM CAT and free Ca^{2+} at the indicated concentrations, and 1 mM ATP was added when indicated. (e) Efflux of ATP from mitochondria was triggered by addition of 10 mM Pi followed by different Ca^{2+} concentrations. The efflux was reverted at the end of the experiment by addition of 1 mM ADP. (f) Ca^{2+} activation of CAT-insensitive ATP transport. Transport rates were calculated from the slopes in panel d after ATP addition and fit to the following equation: $V = V_0 + [(V_{\text{max}} - V_0) \times [\text{Ca}^{2+}]^N / (S_{0.5}^N + [\text{Ca}^{2+}]^N)]$ (where V is transport activity at each $[\text{Ca}^{2+}]$; V_0 is the basal transport rate at $[\text{Ca}^{2+}] \approx 0$; V_{max} is the maximal activity; N is the hill coefficient; and $S_{0.5}$ is the Ca^{2+} concentration, which generates half-maximal transport activity). Pooled data from six independent experiments are shown

Figure 1 Expression of SCaMC isoforms in normal and tumour tissues. Generation of SCaMC-1-KD cell lines. (a) Immunoblot analysis of SCaMC-1 and SCaMC-3 expression in total homogenates from mouse tissues and from COS-7 and 143B cells. Antibodies against Hsp60 were used as loading control. B, brain; L, liver; FL, fetal liver; S, spleen; K, kidney; H, heart; C, colon; SI, small intestine; LU, lung; M, muscle; T, testis; WF, white fat; BF, brown fat. (b) Disease summary for the SCaMC family (SCaMC-1, SCaMC-2, SCaMC-3) analysed by the Oncomine database (www.oncomine.org). The table shows the number of significant unique analyses across the whole Oncomine database (including 93 data sets curated for cancer versus normal analysis), with overexpression (red) or under-expression (blue). Cell colour is determined by the best gene rank percentile for the analyses within the cell. SCaMC-1 shows an overexpression/under-expression ratio of 67/33 from 192 analyses, in contrast to 14/28 from 125 and 19/65 from 165 for SCaMC-2 and SCaMC-3, respectively. A threshold significance value of 0.05 was used including setting to 'ALL' for fold change, gene rank thresholds and data types. For links to the analyses see Supplementary Information. Right panel: Comparison of SCaMC-1/SLC25A24 expression across 67 cancer versus normal analyses, including overexpression (mRNA) and copy-number gain (DNA). The rank for a gene is the median rank for that gene across each of the analyses. The P -value of the median-ranked analysis is shown. For references and link to analysis see Supplementary Information. (c) Protein expression profiles of SCaMC-1 (upper panel) and SCaMC-2 (lower panel) obtained from the Human Protein Atlas database (www.proteinatlas.org) based on immunohistochemistry of cancer samples.⁴⁰ The colour determines the percentage of cancer samples in the database with a given expression level. SCaMC-3 is not available in the database. (d–f) Immunoblot to detect SCaMC-1 and other SCaMC isoforms in total homogenates (d) or isolated mitochondria (e) from COS-7 and 143B control (C) and SCaMC-1-KD (KD) cells. Mouse brain (B) and liver (L) mitochondria are also shown in panel e. Antibodies against Hsp60, GAPDH or CyP-D were used as loading control. (f) Quantification of expression of SCaMC-1 or Hsp60 as compared with GAPDH (mean $\pm$ S.E.M., $n = 5$). (g) Immunofluorescence in COS-7 and 143B control and SCaMC-1-KD cells using anti-SCaMC-1 antibodies

Figure 3 ScaMC-1 selectively protects from oxidative stress-induced cell death. **(a)** Left panel: Control and ScaMC-1-KD 143B cells were incubated with H₂O₂ for 6 h and cell death was evaluated by confocal microscopy after 1 μM calcein-AM and 2 μM PI staining. Right panel: Control and ScaMC-1-KD COS-7 cells were incubated with menadione for 2 h and cell death was evaluated by flow cytometry after PI staining (mean ± S.E.M., *n* = 6 and 5, respectively; **P* < 0.05). **(b)** Control and ScaMC-1-KD 143B cells were incubated with 1 mM H₂O₂ for 3.5 h or with 100 μM menadione for 1 h and cell death was evaluated by flow cytometry after TMRM and Annexin-V-FITC staining. A representative experiment is shown where H indicates the percentage of healthy cells (positive for TMRM and negative for Annexin-V, delimited by the quadrant) and D indicates the percentage of dead cells (negative for TMRM and positive for Annexin-V). The number at the bottom of each panel indicates the percentage (mean ± S.E.M., *n* = 6) of healthy cells. ***P* = 0.00031; **P* = 0.014. **(c)** Control and ScaMC-1-KD COS-7 and 143B cells are equally sensitive to 1 μM staurosporine-induced cell death. Cells were treated with the drug for 0, 16 or 40 h, and death was evaluated by flow cytometry after PI and Annexin-V-FITC staining. **(d)** ScaMC-1-KD cells are more vulnerable to phototoxicity-induced oxidative stress. Control and ScaMC-1-KD COS-7 cells were loaded with 100 nM TMRM; illuminated with 10% laser power of the 543 nm He-Ne line of the confocal system; and collapse of mitochondrial ΔΨ was monitored over time. Lower left panels: ScaMC-1-KD cells were treated with 100 μM EGTA and 1 μM thapsigargin. Lower right panels: Cells treated with 5 μM CsA and 50 μM BKA. The figure shows representative images of at least three independent experiments. **(e)** Sensitivity of ScaMC-1-overexpressing 143B ScaMC-1-KD cells (left panel) and liver clone-9 cells (right panel) to mPT-dependent cell death. Cells were transiently transfected with GFP, or with GFP and ScaMC-1, and exposed to C₂ ceramide or H₂O₂ for 6 h. The bar shows the change in the percentage of GFP fluorescent cells (as compared with total cell number) after the treatment.²¹ An increase in this percentage as compared with control transfections indicates protection by ScaMC-1. The right panel shows the increase in ScaMC-1 expression after the transfection. ScaMC-1*: Rescue construct with synonymous mutations (see the Supplementary Materials and Methods). **(f)** Control and stable ScaMC-1-overexpressing liver clone-9 cells were incubated with H₂O₂ for 3 h and cell death was evaluated by flow cytometry after PI staining (mean ± S.E.M., *n* = 3; **P* < 0.05). The right panel shows the increase in ScaMC-1 expression in the stable ScaMC-1-overexpressing clone

(cytAEQ) or a mitochondrial aequorin (mtAEQwt) probe, respectively.²²

After reconstitution of the probe with the aequorin cofactor coelenterazine, COS-7 cells were stimulated with 100 μ M ATP, an agonist acting on G-protein-coupled receptors and leading to the production of inositol-1,4,5-trisphosphate (IP₃). The consequent Ca²⁺ release from the IC stores induced a cytosolic Ca²⁺ transient, which was identical in control and SCaMC-1-KD cells (Figure 4a, left panel). However, intriguingly, mitochondrial Ca²⁺ uptake was increased almost three times in SCaMC-1-KD cells as compared with controls (Figure 4b, left panel). Similar results were obtained when parental and SCaMC-1-KD 143B cells were stimulated with 100 μ M histamine (Figures 4a and b, right panels).

Mitochondrial free [Ca²⁺]_m is determined by the balance between Ca²⁺ influx into and Ca²⁺ extrusion from the organelle, as well as by Ca²⁺ buffering in the matrix. Thus, in order to characterize the mechanism by which SCaMC-1 activity affects [Ca²⁺]_m, we determined mitochondrial Ca²⁺ influx and efflux rates in isolated mitochondria or permeabilized cells. When measured in the mitochondrial matrix in permeabilized cells expressing the low-affinity mitochondrially targeted aequorin probe (mtAEQmut), addition of the same amount of extra-mitochondrial Ca²⁺ (10 μ M) caused significantly greater elevation in [Ca²⁺]_m in both COS-7 and 143B SCaMC-1-KD cells as compared with their parental counterpart (Figure 4c). However, when measured in the extra-mitochondrial space using the fluorescent Ca²⁺-sensitive dye Calcium-Green, mitochondria from both the parental and SCaMC-1-KD cells took up the same amount of Ca²⁺ with the same rate (Figure 4d). Ca²⁺ efflux was measured after inhibition of Ca²⁺ uptake using RR. In that situation, the rate of Ca²⁺ efflux depends only on the matrix free [Ca²⁺]. After loading 143B mitochondria with Ca²⁺ in the presence of 1 mM ATP-Mg, RR was added. Figure 4e shows that efflux of Ca²⁺ (both Na⁺-independent and Na⁺-dependent) was more rapid in SCaMC-1-KD mitochondria than in controls, indicating (i) higher free [Ca²⁺]_m after Ca²⁺ load in mitochondria lacking SCaMC-1-mediated ATP uptake and (ii) normal activity of the efflux machineries. These results show that increased [Ca²⁺]_m in the mitochondria of SCaMC-1-KD cells is not the consequence of increased Ca²⁺ uptake or reduced Ca²⁺ efflux, but rather reflects a reduced Ca²⁺ buffering capacity in the matrix.

Ca²⁺ buffering in the mitochondrial matrix is principally achieved by the formation of insoluble Ca²⁺-Pi precipitates.²³ A higher level of Ca²⁺ precipitation in control mitochondria could be explained if the adenine nucleotides transported by SCaMC-1 facilitate the precipitation of Ca²⁺ and Pi in the matrix, as has been suggested previously.²⁴ Formation of Ca²⁺-Pi precipitates can be measured by the apparent mitochondrial contraction (an increase in light scattering measured as absorbance at 540 nm) immediately after Ca²⁺ uptake.^{23,25} Indeed, mitochondria of 143B cells showed apparent contraction when Ca²⁺ was added to the medium, which was inhibited by the addition of RR, preventing Ca²⁺ entry through the MCU. Moreover, the initial increase in absorbance in the presence of ATP-Mg was significantly higher in the mitochondria of control than that of SCaMC-1-KD 143B cells (0.041 $\pm$ 0.006 absorbance units per minute

compared with 0.018 $\pm$ 0.003, respectively, mean $\pm$ S.E.M., $n=3$, $P=0.048$; not shown). These results suggest a higher efficiency of Ca²⁺-Pi precipitate-mediated Ca²⁺ buffering in the presence of SCaMC-1.

SCaMC-1 increases mitochondrial CRC and reduces the probability of Ca²⁺-mediated mPTP opening. Regulation of Ca²⁺-dependent mPTP opening by low micromolar concentrations of ATP/ADP is well established,²⁶ and is mediated by a nucleotide-induced switch in the conformation of ANT. However, higher concentrations of adenine nucleotides, in the physiological millimolar range, are also able to prevent mPTP formation.²⁷ Thus we tested whether Ca²⁺-mediated uptake of ATP-Mg/ADP by SCaMC-1 can affect mitochondrial CRC, a measure of the amount of Ca²⁺ required in order to trigger mPT.

CRC was studied by monitoring Ca²⁺ uptake into mitochondria by using Calcium-Green to measure [Ca²⁺]_m in the buffer. Permeabilized cells were treated with sequential Ca²⁺ pulses and Ca²⁺ uptake into mitochondria was measured as a decrease in fluorescence. mPTP opening was detected as a steady increase of fluorescence owing to Ca²⁺ release from mitochondria. Other organelles, such as the ER, do not contribute significantly to Ca²⁺ uptake, as RR completely inhibited uptake (not shown). As summarized in Figure 5a, permeabilized control and SCaMC-1-KD COS-7 cells show similar mitochondrial CRCs both in the absence of adenine nucleotides and in the presence of low ADP (100 μ M). In both lines, 100 μ M ADP increased the CRC only slightly (less than 10% on COS-7 cells ($P>0.05$); 20% in 143B cells ($P<0.05$); not shown), probably by interacting with the ANT and triggering the 'm' conformation of the carrier, as demonstrated previously in liver and brain mitochondria.²⁰ Most importantly, however, in the presence of 1 mM ATP or 2 mM ADP, control COS-7 or 143B cells showed a pronounced increase in the threshold for mPTP opening as compared with SCaMC-1-KD cells, whose CRC was practically identical to that measured in the presence of 100 μ M ADP. The presence of Mg²⁺ was essential for the protective effect of ATP, but not for that of ADP, consistently with the substrate specificity of SCaMC-1. The same results were obtained in isolated mitochondria from COS-7 or 143B cells (Figures 5b and c), as mitochondria from control cells are able to take up more Ca²⁺ in the presence of 1 mM ATP or 2 mM ADP than SCaMC-1-KD mitochondria before release of accumulated Ca²⁺ by mPTP opening.

mPTP opening can also be studied by monitoring mitochondrial swelling. Figure 5d shows that Ca²⁺ addition to COS-7 mitochondria in the presence of ATP initially caused contraction, while further additions led to swelling (a decrease in absorbance). The number of Ca²⁺ additions needed to trigger swelling in the presence of 2 mM ATP was always higher in control mitochondria as compared with SCaMC-1-KD mitochondria. On the other hand, swelling was identical in the absence of nucleotides. Similar results were obtained in 143B isolated mitochondria (Figure 5e).

In the presence of 5 μ M CsA, an inhibitor of mPT, the CRC was enhanced in permeabilized cells and isolated COS-7 mitochondria (Figures 5a and b), and mitochondrial swelling was inhibited (Figure 5d), demonstrating that the effects

observed were due to mPTP opening. CsA produced these effects in both control and ScaMC-1-KD mitochondria, but differences between both lines in the presence of 1 mM ATP

were still observed. On the other hand, 5 μ M CAT, a stimulator of mPT, greatly reduced the CRC in both control and ScaMC-1-KD permeabilized cells, but a difference between the two

Figure 5 SCaMC-1 reduces the Ca^{2+} sensitivity of mPT in isolated mitochondria and permeabilized cells. Ca^{2+} -mediated mPT in control (black traces) and SCaMC-1-KD (red traces) COS-7 (a, b and d) and 143B cell (c and e) mitochondria. (a) Quantification of CRC after administration of subsequent Ca^{2+} pulses (20 μM each) to control (white bars) and SCaMC-1-KD (black bars) permeabilized COS-7 cells (0.15 mg total protein per millilitre). CRC was determined in the presence or absence of adenine nucleotides, Mg^{2+} and inhibitors. The Ca^{2+} concentration required to open the mPTP is shown (mean $\pm$ S.E.M., $n = 6-16$). (b and c) CRC was measured in isolated mitochondria in the presence of 1 mM ATP (b) or 2 mM ADP (c) by using the Ca^{2+} -sensitive probe Calcium-Green 5N free salt in the extra-mitochondrial space. Experiments started by addition of mitochondria, followed by repetitive Ca^{2+} pulses (arrows, 20 μM each). The traces marked by a long arrow are in the presence of 5 μM CsA. (d and e) Swelling of isolated mitochondria after subsequent Ca^{2+} pulses (arrows, 40 μM each) was measured as a decrease in light scattering (as absorbance at 540 nm) in the presence (left panel) or absence (middle panel) of 2 mM ATP. Traces in the right panel were recorded in the presence of 2 mM ATP and either 5 μM CsA (d) or 200 nM RR (e). (f) The ERK pathway is constitutively active in 143B cells, but not in COS-7 cells, and controls CyP-D phosphorylation. Left panels: Immunoblot against phospho-ERK and total ERK, and phospho-GSK-3 β and total GSK-3 β , in total homogenates from COS-7 and 143B cells. Right panel: Immunoblot against CyP-D in total homogenates from control or FLAG-CyP-D⁸ overexpressing COS-7 and 143B cells after Mn^{2+} -Phos-tag SDS-PAGE.³⁹ Phosphorylated proteins are shifted upwards in the gel compared with unphosphorylated proteins. The same bands were observed when anti-FLAG antibodies were used, confirming the specificity of the bands (not shown). CyP-D phosphorylation is greatly decreased if the extracts are treated with alkaline phosphatase (AP) for 30 min (far right lane)

Figure 4 SCaMC-1 knock-down reduces mitochondrial Ca^{2+} buffering in intact cells and isolated mitochondria. Agonist-induced Ca^{2+} increases in the cytosol (a) and mitochondria (b) of cells transiently expressing cytAEQ and mtAEQwt probes, respectively. Ca^{2+} signals were induced by ATP in COS-7 and by histamine in 143B cells (black, controls; red, SCaMC-1-KD). The bar charts show quantification of results in control (C) and SCaMC-1-KD cells (KD, mean $\pm$ S.E.M., $n = 3$). (c) $[\text{Ca}^{2+}]_m$ of COS-7 (left panel) and 143B (right panel) permeabilized cells after addition of 10 μM (free $[\text{Ca}^{2+}]$) Ca^{2+} to the IC buffer. The bar charts show quantification of results in control (C) and SCaMC-1-KD cells (KD, mean $\pm$ S.E.M., $n = 3$). (d) Ca^{2+} uptake rate in permeabilized cell and isolated mitochondria, measured by using the Calcium-Green 5N free salt as an extra-mitochondrial Ca^{2+} indicator. A 20- μM (free $[\text{Ca}^{2+}]$) Ca^{2+} pulse was added where indicated (black, controls; red, SCaMC-1-KD). (e) Ca^{2+} efflux was measured by using the Calcium-Green 5N free salt in the extra-mitochondrial space of isolated 143B mitochondria, in the presence of 1 mM ATP, followed by loading with 320 nmol of Ca^{2+} per mg of protein. Where indicated, 200 nM RR was added to inhibit Ca^{2+} uptake, followed by addition of 10 mM NaCl to stimulate the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (black, controls; red, SCaMC-1-KD). The bar chart shows quantification of Na^+ -independent (white bars) and Na^+ -dependent (black bars) Ca^{2+} efflux rates in control and SCaMC-1-KD mitochondria (mean $\pm$ S.E.M., $n = 3$; $*P < 0.05$)

cell lines in the presence of 1 mM ATP was still observed (Figure 5a). The increase in CRC by 1 mM ATP in the presence of CAT in control cells proves that the effect of high concentrations of adenine nucleotides was indeed independent of the ANT, as had been suggested previously.²⁷ Furthermore, in the presence of 5 μ M BKA, another ANT inhibitor, the differences between both lines obtained at 1 mM ATP were still present (Figure 5a).

Interestingly, 143B permeabilized cells and isolated mitochondria were largely unaffected by CsA (Figure 5c and Supplementary Figure S4B). This is not due to a lower expression of CyP-D (Figure 1e), the regulatory component of the pore that is targeted by CsA, an argument, which is used to explain the CsA insensitivity of brain mitochondria.²⁸ An alternative explanation is that 143B are tumour cells, whereas COS-7 are merely immortalized cells. Tumour cells show a constitutive activation of the ERK pathway, which has been suggested to dephosphorylate and inactivate CyP-D,⁸ and, thus, although present, CyP-D may be inactive in 143B mitochondria. In order to study this possibility, first the phosphorylation state of CyP-D was assayed by the Mn²⁺-Phos-tag SDS-PAGE technique. In FLAG-CyP-D-overexpressing COS-7 cells, several shifted CyP-D bands were observed, demonstrating the phosphorylated status of the protein (Figure 5f). Importantly, these bands were almost completely missing in FLAG-CyP-D-overexpressing 143B cells in spite of equal expression of CyP-D. In addition, 143B cells showed a constitutive phosphorylation of ERK, whereas COS-7 cells did not (Figure 5f). In this pathway, ERK was shown to phosphorylate and inactivate GSK-3 β , the putative CyP-D kinase. Indeed, we found that GSK-3 β was phosphorylated in 143B cells but not in COS-7 cells (Figure 5f). These results suggest that, in 143B cells, as a result of a constitutively active ERK/GSK-3 β pathway, CyP-D is mostly dephosphorylated and thus inactive. Conversely, in COS-7 cells CyP-D is partially phosphorylated and active owing to lack of ERK activation.

In summary, these results provide evidence that adenine nucleotides regulate mPTP opening independently of the ANT, through a lower affinity site, reflecting the activity of the ATP-Mg/Pi carrier. Modification of the mitochondrial adenine nucleotide content by the ATP-Mg/Pi carrier regulates the CRC and mPTP opening, probably by modifying the formation of Ca²⁺-Pi precipitates and thus altering the Ca²⁺ buffering capacity of the mitochondrial matrix.

Concluding Remarks

Altogether, our data fully support a model where SCaMC-1 is responsible for a cytoprotective mechanism by mediating (i) ATP/ADP uptake in the mitochondrial matrix, triggered by increases of [Ca²⁺]_{cyt}; (ii) increased mitochondrial Ca²⁺ buffering capacity owing to elevated adenine nucleotide levels; and (iii) desensitization of Ca²⁺-mediated mPT. This pathway represents a novel mechanism promoting cancer cell survival under stress conditions.

High levels of SCaMC-1 were previously found by screening approaches in colorectal carcinomas,²⁹ breast cancer³⁰ and in several cancer cell lines.^{15,31} Our *in silico* studies confirmed and extended these findings to 67 different tumour

types where SCaMC-1 expression significantly exceeded its expression in their normal tissue counterpart. Importantly, this pattern was specific to the SCaMC-1 isoform, which has an order of magnitude higher transport activity than SCaMC-3.¹⁸ This suggested that the benefit conferred by SCaMC-1 overexpression implies a high rate of transport of adenine nucleotides in the mitochondrial matrix under conditions of pathologically elevated [Ca²⁺]_{cyt}. The S_{0.5} for Ca²⁺ of SCaMC-1 was found to be 12.7 μ M, adequate to respond to changes in the [Ca²⁺]_{cyt} that are transmitted to the mitochondria through the MCU during Ca²⁺ overload both under physiological and pathological conditions. Indeed, SCaMC-1 expression was not essential for cellular proliferation, rather was required for protection against oxidative stress- and Ca²⁺ overload-induced cell death. This resembles the effect of Bcl-2 overexpression,³² Bax and Bak downregulation,⁶ Bad phosphorylation,³³ hexokinase-II binding to mitochondria⁷ and constitutive activation of the ERK pathway.⁸ Distinct from some of these studies, however, is that the role of SCaMC-1 was exclusive to mPT-driven cell death, as no effect was observed on cell death induced by staurosporine. Altogether, selective overexpression of SCaMC-1 over normal tissues and its specific role in protection from oxidative stress-induced cell death indicate that selective ablation of SCaMC-1 function might represent a novel strategy to abolish tumour growth in a wide range of cancers.

Low concentrations of adenine nucleotides ($\approx$ 100 μ M) inhibit mPTP opening in isolated liver and brain mitochondria,²⁶ most likely by conformational changes triggered by binding to high-affinity sites ($K_d \approx$ 5–10 μ M) of the ANT.²⁰ However, adenine nucleotides in the millimolar, physiological range also show potent mPT inhibition, even in the presence of ANT inhibitors.^{10,27,34} The site of this ANT-independent protection remained unexplained.³⁵ Here we found that SCaMC-1, which shows binding sites for adenine nucleotides in this affinity range ($K_d \approx$ 0.2–0.5 mM), is able to mediate this effect.

While the mechanism by which matrix adenine nucleotides protect from mPT is not completely understood, our data clearly suggest that altered mitochondrial Ca²⁺ handling has a major role in the effect. The lack of ATP/ADP uptake in the mitochondria of SCaMC-1-KD cells led to a vast increase in free matrix [Ca²⁺] after activation of cellular Ca²⁺ signalling pathways, as compared with cells with high SCaMC-1 expression levels.

Accordingly, we propose that the SCaMC-1-mediated adenine nucleotide load in the mitochondrion buffers matrix free [Ca²⁺], preventing mitochondrial Ca²⁺ overload during oxidative stress and preventing mPT induction. As a major route of mitochondrial Ca²⁺ buffering, Ca²⁺ and Pi form amorphous Ca₃(PO₄)₂ precipitates in the matrix.^{23,25} It has long been suggested that presence of adenine nucleotides in the mitochondrial matrix is essential for this process, either by stabilizing the Ca²⁺-Pi deposits or by priming their precipitation.²⁴ As a result, mitochondrial Ca²⁺-Pi precipitates contain adenine nucleotides,³⁶ and ATP may account for as much as 7% of the dry weight of some Ca²⁺-Pi precipitates.³⁷ Interestingly, Ca²⁺-Pi precipitates in isolated mitochondria also contain Mg²⁺ when Ca²⁺ loading was performed in

the presence of ATP, but not in the presence of ADP,²⁵ which matches the substrate specificity of the ATP-Mg/Pi carriers.^{11,18}

The protective effect of increasing amounts of matrix Ca^{2+} -Pi precipitates has already been observed, particularly in cell death models mediated entirely by Ca^{2+} overload, such as glutamate excitotoxicity in primary neurons.³⁸ Our findings extend these observations to cancer cells, and provide direct demonstration of the effect of SCaMC-1 knockdown-mediated adenine nucleotide depletion on mitochondrial Ca^{2+} handling.

Materials and Methods

Cell culture, transfection, generation of SCaMC-1-KD and mitochondrial luciferase-expressing clones. COS-7 cells, liver clone-9 cells and 143B osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere. For transfections, the LipofectAMINE reagent (Invitrogen) was used as described previously.¹⁵ Details of the constructs and generation of SCaMC-1-KD clones, cells overexpressing SCaMC-1 or cells stably expressing mitochondrially targeted luciferase are described under Supplementary Materials and Methods.

Immunofluorescence and western blotting. Immunofluorescent stainings were performed as described previously.¹⁵ Anti-SCaMC-1 antibodies were diluted 1:400. Fluorescence microscopy was performed by using an Axiovert epifluorescence microscope (Carl Zeiss, Göttingen, Germany) at a nominal magnification of $\times 100$.

For western blotting, total homogenates and mitochondrial-enriched fractions were obtained from cells and mouse tissues as described¹⁵ and analysed by western blotting (for details see Supplementary Materials and Methods). To determine the phosphorylation status of CyP-D, Mn^{2+} -Phos-tag SDS-PAGE was performed.³⁹ Polyacrylamide gels were supplemented with 200 μM MnCl_2 and the Phos-tag reagent (Nard Institute, Amagasaki, Japan). Phosphorylated proteins bind to the Phos-tag, and thus are delayed in the gel and separated from unphosphorylated proteins. After electrophoresis, the gels were washed with transfer buffer containing 1 mM EDTA and then with transfer buffer without EDTA according to the manufacturer's protocol, before transfer to PVDF membranes. The membranes were probed with CyP-D antibodies.

Measurements of mitochondrial Ca^{2+} uptake and swelling on isolated mitochondria or permeabilized cells. Mitochondria were isolated by homogenization and differential centrifugation according to standard protocols. $[\text{Ca}^{2+}]_m$ uptake was measured in the presence of the Ca^{2+} -sensitive fluorescent probe Calcium-Green 5N (0.1 μM , excitation 506 nm, emission 532 nm) using digitonin-permeabilized cells or isolated mitochondria (for details see Supplementary Materials and Methods).

Measurement of cellular respiration and $\Delta\psi_m$. Cell respiration was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA; for details see Supplementary Materials and Methods). $\Delta\psi_m$ was measured using the fluorescent lipophilic cationic dye TMRM. Cells were stained with 200 nM TMRM for 10 min and red fluorescence was measured by flow cytometry.

Ten percent laser power of the 543 He-Ne laser line on the Zeiss 510 confocal system was used to induce mPT, which was detected by loss of TMRM signal, as described previously.¹⁹ Cells were pre-incubated with 100 nM TMRM for 20 min at 37 °C in a modified Krebs-Ringer buffer (KRB; 135 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 0.4 mM K_2HPO_4 , 1 mM CaCl_2 , 5.5 mM glucose, 20 mM HEPES, pH 7.4). TMRM was then present throughout the subsequent measurements. Confocal images were obtained using a Zeiss 510 LSM/META system, using a $\times 40$ oil-immersion objective. For detection a 560-nm long-pass filter was used.

Luminescent detection of cellular $[\text{Ca}^{2+}]$ and [ATP]. $[\text{Ca}^{2+}]_{cyt}$ and $[\text{Ca}^{2+}]_m$ in intact cells were measured 48 h after transient transfection with cytAEQ and mtAEQwt probes as described previously.²² To measure mitochondrial ATP levels in permeabilized cells, experiments were performed 24 h after transient transfection with mitochondrial luciferase.¹⁷ For experimental details see Supplementary Materials and Methods.

Cell death detection. Cells were incubated with H_2O_2 , menadione or staurosporine for the time periods indicated in the figure legends and cell death was evaluated by previously described methods:^{5,7,8,21} (i) by microscopy after loading with 1 μM calcein-AM to stain live cells and 2 μM PI to stain the nuclei of dead cells; (ii) by flow cytometry after loading with 2 μM PI to stain the nuclei of dead cells; (iii) by flow cytometry after incubation with 200 nM TMRM to detect mitochondrial depolarization and FITC-conjugated Annexin-V (Sigma, St. Louis, MO, USA) to detect phosphatidylserine exposure on the cell surface; and (iv) by flow cytometry using the Annexin-V-FITC Apoptosis Detection kit (Sigma).

Conflict of Interest

The authors declare no conflict of interest.

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