Metabolic adaptation to the chronic loss of Ca\textsuperscript{2+} signaling induced by KO of IP\textsubscript{3} receptors or the mitochondrial Ca\textsuperscript{2+} uniporter

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Calcium signaling is essential for regulating many biological processes. Endoplasmic reticulum inositol trisphosphate receptors (IP\textsubscript{3}R), and the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) are key proteins that regulate intracellular Ca\textsuperscript{2+} concentration. Mitochondrial Ca\textsuperscript{2+} accumulation activates Ca\textsuperscript{2+}-sensitive dehydrogenases of the tricarboxylic acid (TCA) cycle that maintain the biosynthetic and bioenergetic needs of both normal and cancer cells. However, the interplay between calcium signaling and metabolism is not well understood. In this study, we used human cancer cell lines (HEK293 and HeLa) with stable KOs of all three IP\textsubscript{3}R isoforms (triple KO [TKO]) or MCU to examine metabolic and bioenergetic responses to the chronic loss of cytosolic and/or mitochondrial Ca\textsuperscript{2+} signaling. Our results show that TKO cells (exhibiting total loss of Ca\textsuperscript{2+} signaling) are viable, displaying a lower proliferation and oxygen consumption rate, with no significant changes in ATP levels, even when made to rely solely on the TCA cycle for energy production. MCU KO cells also maintained normal ATP levels but showed increased proliferation, oxygen consumption, and metabolism of both glucose and glutamine. However, MCU KO cells were unable to maintain ATP levels and died when relying solely on the TCA cycle for energy. We conclude that constitutive Ca\textsuperscript{2+} signaling is dispensable for the bioenergetic needs of both IP\textsubscript{3}R TKO and MCU KO human cancer cells, likely because of adequate basal glycolytic and TCA cycle flux. However, in MCU KO cells, the higher energy expenditure associated with increased proliferation and oxygen consumption makes these cells more prone to bioenergetic failure under conditions of metabolic stress.

Ca\textsuperscript{2+} signaling plays an important role in regulating many diverse biological processes ranging from cell proliferation, secretion, motility, metabolism, and cell death (1). A key event that initiates Ca\textsuperscript{2+} signaling is the release of Ca\textsuperscript{2+} from intracellular stores by the three isoforms of inositol trisphosphate receptor (IP\textsubscript{3}R) channels (2). The depletion of internal Ca\textsuperscript{2+} stores secondarily stimulates Ca\textsuperscript{2+} entry via Orai channels in the plasma membrane. Thus, loss of IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} signaling would block both the intracellular and extracellular components of agonist-mediated Ca\textsuperscript{2+} signaling. An increase of cytosolic Ca\textsuperscript{2+} in the environment of the mitochondria facilitates Ca\textsuperscript{2+} uptake into the matrix by a complex of inner membrane proteins of which the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) is the pore-forming constituent (3). The elevations of Ca\textsuperscript{2+} in the cytosol and mitochondrial compartments act on multiple effector proteins to alter cell function (1, 4). In the mitochondrial matrix, Ca\textsuperscript{2+} stimulates the activity of three dehydrogenases of the tricarboxylic acid (TCA) cycle (5), respiratory complexes (6), and ATP synthase (7) to enhance ATP generation. Higher matrix Ca\textsuperscript{2+} accumulation can open the permeability transition pore and cause cell death (8). Inactivating the function of all three IP\textsubscript{3}R isoforms or MCU channels would therefore be expected to have wide-ranging effects, including compromising the metabolic and bioenergetic status of the cells.

Global and conditional KO mouse models of individual IP\textsubscript{3}R isoforms have provided valuable information on the role of Ca\textsuperscript{2+} signaling in regulating organ function (9–14). However, functional redundancy and postnatal mortality have complicated these studies (15–19). Few studies have examined the effects of the conditional deletion of all three IP\textsubscript{3}R isoforms. In hematopoietic cells, these investigations indicated a critical role for Ca\textsuperscript{2+} in regulating early developmental checkpoints in T and B lymphocytes (20, 21). In mouse embryonic stem cells, the IP\textsubscript{3}R TKO cells showed enhanced differentiation into the cardiomyocyte lineage and impaired differentiation into the hematopoietic lineage (22). Inducible KOs of all IP\textsubscript{3}R isoforms in mouse vascular endothelial cells (14) or smooth muscle cells (13) have been shown to affect basal blood pressure and vascular tone.

Studying the mechanisms of adaptation to a loss of Ca\textsuperscript{2+} signaling is much easier in cultured cell systems than in animal
KO models. A widely used cell culture model is a chicken DT40 cell line in which all three IP3R isoforms have been disrupted (23). However, studies using DT40 cells have primarily focused on the structure, function, and regulation of IP3Rs (24). A number of reports have noted that DT40 IP3R TKO cells have a baseline increase in autophagy (25–27). Cardenas et al. (27) proposed that the increased autophagy compensated for the bioenergetic deficit caused by the loss of endoplasmic reticulum (ER)/mitochondrial Ca^{2+} signaling required to maintain optimal operation of the TCA cycle. Cardenas et al. (28, 29) have extended these studies to compare the effects of acute inhibition of ER/mitochondrial Ca^{2+} transfer in normal and cancer cells. They concluded that, unlike normal cells, the stimulation of autophagy in cancer cells is not sufficient to compensate for the increased energy demand of uncontrolled growth, with cell death resulting from a “bioenergetic crisis” during mitosis. The advent of new methods of gene disruption has enabled all three IP3R isoforms to be deleted in human embryonic kidney 293 (HEK293) (30, 31) and HeLa (32) human cancer cell lines. While these KO cells have primarily been used as an expression system to study the functional properties of IP3R mutants, they also offer the opportunity to investigate the metabolic and bioenergetic consequences of the chronic effects of a total loss of Ca^{2+} signaling. This is the primary objective of the present investigation.

Many studies have examined the physiological consequences of disrupting mitochondrial Ca^{2+} signaling using global and tissue-specific MCU KO mouse models. In most cases, a relatively mild phenotype at baseline has been observed in tissues, such as the heart (33), skeletal muscle (34), exocrine pancreas (35), endocrine pancreas (36), brown adipose tissue (37), and photoreceptors (38) suggesting the existence of robust adaptive mechanisms that have yet to be fully characterized, including upregulation of alternative mechanisms of mitochondrial Ca^{2+} transport. Where metabolic changes have been investigated in MCU KO cells, the results have generally been tissue specific. For example, fatty acid oxidation is increased in cardiac (39) and skeletal muscle (34), and decreased in liver (40) and pulmonary macrophages (41).

Relatively few studies have examined the metabolic or bioenergetic consequences of knockouting MCU in cultured cells. Using RNA interference, it was concluded that MCU was dispensable for growth in a breast cancer cell line (42) but stimulated the growth and metastasis of hepatocellular carcinoma (43) and colorectal cancer cells (44). Silencing of MCU in mouse pancreatic β-cells suppressed the increase of ATP/ADP induced by glucose (45). The absence of MCU in vascular smooth muscle cells impaired mitochondrial fusion and entry into the G1/S phase of the cell cycle (46). The conditional deletion of MCU from mouse embryonic fibroblasts (MEFs) enhanced differentiation to myofibroblasts by an epigenetic mechanism that involves enhanced glycolysis and increased accumulation of α-ketoglutarate (α-KG) (47). Overall, it is clear that the metabolic phenotype of the MCU KO cells is dependent on the cell type being investigated. To obtain a clearer picture of the metabolic consequences of disrupting either ER or mitochondrial Ca^{2+}, we have carried out the present study in which IP3Rs or MCU have been knocked out in the same human cancer cell lines. Our studies show that the IP3R TKO cells have adapted to the total loss of Ca^{2+} signaling with a lower proliferation and oxygen consumption rate (OCR), but no significant changes in adenine nucleotides, even when artificially made to rely entirely on the TCA cycle for their bioenergetic and biosynthetic needs. By contrast, the loss of MCU is associated with an increased proliferation and OCR, increased metabolism of glucose and glutamine, a higher level of basal AMP-dependent kinase (AMPK), and bioenergetic failure when made to rely solely on the TCA cycle. We conclude that baseline rates of aerobic glycolysis and the TCA cycle are sufficient to make constitutive ER/mitochondrial Ca^{2+} signaling dispensable for the growth and function of both IP3R TKO and MCU KO cancer cells. However, in MCU KO cells, the higher energy expenditure associated with increased proliferation and oxygen consumption make these cells more prone to bioenergetic failure under conditions of metabolic stress.

**Results**

**Cytosolic and mitochondrial Ca^{2+}**

We initially characterized cytosolic Ca^{2+} signals in the IP3R and MCU KO HEK293 cell models incubated in the absence of extracellular Ca^{2+}. As expected, the loss of all three IP3R isoforms prevented any cytosolic Ca^{2+} changes induced by carbachol (Cch) stimulation of endogenous muscarinic receptors (Fig. 1, A and B). MCU deletion was carried out in the variant HEK293T cell line (48), and these cells showed a decreased Cch-mediated cytosolic Ca^{2+} signal when compared with the appropriate control of WT HEK293T cells. The cytosolic Ca^{2+} responses of WT HEK293 and HEK293T cells were not significantly different (Fig. 1A), and this also proved to be the case for several other parameters measured in this study. This includes cell growth (Fig. 2A), glucose utilization (Fig. S1A), glutamine consumption (Fig. S1B), total NAD⁺ and NADH (Fig. S1, C and D), lactate accumulation (Fig. S1E), ATP levels (Fig. S1F), phosphorylated AMPK levels (Fig. S1G), and phosphorylated pyruvate dehydrogenase (p-PDH) levels (Fig. S1H). Hence, only the WT HEK293 cells are represented as controls in most of the remaining figures.

The ability of mitochondria to sequester and release a 50 μM pulse of Ca^{2+} in saponin-permeabilized cell lines was measured in Figure 1, C and D. Both WT and IP3R TKO cells had comparable rates of mitochondrial uptake and (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) (FCCP)-induced release compared with the negligible rates for both processes seen in the MCU KO cells. The MCU KO–permeabilized cells consistently showed an elevated steady state [Ca^{2+}] in the medium prior to Ca^{2+} pulse addition. This may reflect the failure of the cells to sequester the endogenous contaminating Ca^{2+} into the mitochondria before the initiation of the experiment. The mitochondrial Ca^{2+} content in intact cells was estimated using the ratiometric, high-
affinity, genetically encoded, fluorescent probe GEM-GECO ($K_d \approx 340$ nM; (49)). The baseline signal in all three cell lines was not significantly decreased by chelation of extracellular Ca$^{2+}$ or by depolarization with FCCP/oligomycin, indicating that there is very little Ca$^{2+}$ in the mitochondria under unstimulated conditions (Fig. 1, E and F). The measured basal content was only marginally lower (~20%) in IP$_3$R TKO and MCU KO cells (Fig. 1F). It should be noted that a variable lowering of mitochondrial Ca$^{2+}$ content has been reported in mouse MCU KO models (50–53). The ability of the probe to register increases in mitochondrial Ca$^{2+}$ was verified by measuring responses to ionomycin and Cch (Fig. 1, E, G, and H). Parallel measurements of cytoplasmic and mitochondrial Ca$^{2+}$ confirmed that Cch-induced increases in mitochondrial Ca$^{2+}$ were suppressed in the MCU KO cells (Fig. 1, G–I). Taken together, the results show that IP$_3$R TKO cells have lost agonist-mediated cytosolic Ca$^{2+}$ signaling but retain the ability to sequester mitochondrial Ca$^{2+}$, whereas the MCU KO cells have retained cytosolic Ca$^{2+}$ signaling (albeit at a reduced level) but have lost
Mitochondrial Ca$^{2+}$ uptake. The impact of these Ca$^{2+}$ signaling phenotypes on metabolism and bioenergetics is investigated later.

**Cell growth and glucose metabolism**

A number of basic growth and metabolic parameters were measured in the HEK293 cell lines (Fig. 2). In the Dulbecco’s modified Eagle’s medium (DMEM) growth medium (containing glucose [25 mM], glutamine [5 mM], and pyruvate [1 mM]), the IP$_3$R TKO cells grew more slowly, and the MCU KO cells grew more rapidly, than their corresponding WT counterpart (Fig. 2A). The cells were entirely dependent on glucose and glutamine as fuels, since the omission of either substrate prevented growth in all the cell lines (not shown). Glucose consumption was measured at a lower glucose concentration of 5 mM and found to be comparable between WT and IP$_3$R TKO cells (Fig. 2B). However, the MCU KO cells consumed glucose more rapidly (Fig. 2B). Both cellular lactate (Fig. 2C) and pyruvate (Fig. 2D) showed increased levels in the MCU KO cells. The assumption of near equilibrium of the lactate dehydrogenase (LDH) reaction allows calculation of the free cytosolic NAD$^+$/NADH ratio from the lactate/pyruvate ratio (54). This calculation indicated a substantial increase in the NAD$^+$/NADH ratio in MCU KO cells (Fig. 2E). To verify these findings, the total cellular levels of NAD$^+$ and NADH were also measured directly by an enzymatic cycling method (Fig. 2F and G). Significant changes in NAD$^+$ levels were not observed, but the levels of NADH decreased substantially in MCU KO cells and to a lesser extent in IP$_3$R TKO cells (Fig. 2G). Similar changes were also observed for NADP$^+$/NADPH (Fig. S2A). Quantitation of the total levels of metabolites by mass spectroscopy showed that MCU KO cells have increased levels of glutamate, $\alpha$-KG, and citrate (Fig. S2B). These metabolite changes are consistent with increased metabolism of glutamine in MCU KO cells. Similar
increases in α-KG have been noted in MCU KO fibroblasts (47) and photoreceptors (38). In fibroblasts, the increase in α-KG was attributed to enhanced glutamine metabolism and linked to epigenetic changes altering the methylation of histones. In photoreceptors, enhanced glutamine metabolism was not observed. Both malate and aspartate levels were decreased in the MCU KO HEK cells (Fig. S2B). The fall in aspartate could in part be related to increased utilization, since large increases were observed in carbamoyl aspartate (used in pyrimidine synthesis) and asparagine levels. The fall in malate is expected, since malate is in equilibrium with aspartate through oxaloacetate. The fall in these metabolites may disrupt the malate/aspartate shuttle and may account for the increased reliance on lactate production as a means of regenerating the NAD+ required for glycolysis. Overall, these results indicate that there are marked increases in growth rate, glycolysis, and NAD+/NADH redox state in the MCU KO cells.

**Key bioenergetic parameters in WT, IP₃R TKO, and MCU KO cells**

Altered supply of NADH by dehydrogenases and/or utilization by the respiratory chain may underlie the observed changes in NAD+/NADH ratio. This prompted us to examine the OCR of the KO cell lines (Fig. 3, A and B). In intact cells exposed to complete growth medium, the basal OCR of MCU KO cells was increased by ~60% relative to WT cells. Under the same conditions, the OCR of IP₃R TKO cells was

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**Figure 3. Some bioenergetic parameters of HEK293 KO cells.** A, equal amounts of cells (10⁶) incubated in complete DMEM were added to the chamber of an O₂ electrode, and rates of O₂ consumption (OCR) were measured under basal conditions and after the sequential additions of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (2 μM). B, quantification of OCR is shown normalized to the basal OCR of WT cells. The data are the mean ± SEM of three independent experiments, each carried out in triplicate: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, two-way ANOVA followed by multiple comparison analysis using Dunnett’s test. C, ATP was measured in neutralized perchloric acid (PCA) lysates with a luciferase assay. D, AMP was measured in lysates processed for LC–MS/MS experiments. E and F, the phosphorylation status of AMPK and PDH measured by immunoblotting with phospho-specific antibodies. Values are normalized to the total levels of the enzymes. Statistical evaluation for (C–F) used one-way ANOVA with symbols as in (B). AMPK, AMP-dependent kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCCP, (carbonylcyanoide-4-(trifluoromethoxy)phenyllhydrazone); HEK293, human embryonic kidney 293T cell line; OCR, oxygen consumption rate; PDH, pyruvate dehydrogenase.
decreased by ~25%. The same trends were observed when respiration was uncoupled with FCCP. Differences in OCR in intact cells could reflect altered availability of substrates to the respiratory chain. However, the decreased OCR in IP$_3$R TKO cells and increased OCR in MCU KO cells was also observed in permeabilized cells supplied with an excess of glutamate/malate and ADP (Fig. S2C). In the case of MCU KO cells, differences of OCR seen in intact cells in the presence of oligomycin or FCCP were not observed in permeabilized cells, suggesting that the differences seen in intact cells under these conditions may reflect enhanced substrate availability. We examined the ability of the cell lines to maintain basal ATP levels and found no statistical differences between the three cell lines (Fig. 3C). However, the basal AMP levels were substantially elevated in the MCU KO cells and to a smaller extent in IP$_3$R TKO cells (Fig. 3D). The different AMP levels correlated with the phosphorylation state of AMPK in the three cell lines (Fig. 3E). PDH is a key mitochondrial phosphoenzyme controlling the flow of glucose carbon into the TCA cycle and would therefore be one of many steps controlling NADH supply. The dephosphorylation and activation of PDH is catalyzed by a Ca$^{2+}$-sensitive phosphatase (5). In agreement with other MCU KO models (34, 50, 55, 56), the phosphorylation of PDH is substantially increased in MCU KO HEK293T cells. However, PDH phosphorylation was unaltered in IP$_3$R TKO cells (Fig. 3F).

Autophagy is a key cellular process regulated by the energy sensor AMPK (57). We therefore measured autophagy in the three cell lines by quantitating microtubule-associated protein 1 light chain 3 (LC3) conversion and p62 degradation detected by immunoblotting (Fig. 4). The data show increased levels of LC3-II (Fig. 4, A and B) and p62 (Fig. 4, C and D) in the IP$_3$R TKO cells but not in MCU KO cells. In order to distinguish between effects on enhanced autophagosome formation or decreased fusion with lysosomes, we blocked the latter process by preincubation of the cells with bafilomycin (58). The increase in LC3-II/p62 observed in IP$_3$R TKO cells was retained in the bafilomycin-treated cells with no additional changes seen in the MCU KO cells. The results are in line with several studies showing enhanced autophagy in IP$_3$R TKO DT40 lymphocytes that is AMPK independent ((25, 26); but see Ref. (27)). The data also show that the AMPK activation seen in MCU KO cells does not lead to enhanced autophagy.

**Isotope tracer analysis**

To investigate the metabolism of glucose in more detail, we used U-13C-glucose and mass spectroscopy. For the isotope
tracer experiments, the glucose in DMEM was reduced to 5 mM for 30 min and then switched to 5 mM U–13C-glucose for 5 and 60 min. We found the labeling of glycolytic intermediates to be the same at both time points, and therefore, only the 60 min data are shown (Fig. 5). The rapid labeling kinetics precluded any conclusions regarding differences in glycolytic rates based on U–13C-glucose experiments. The fractional enrichment (FE) of m + 5 ribose-5-phosphate was decreased in MCU KO cells suggesting that less glucose carbon may be diverted into the pentose phosphate shunt in these cells (Fig. 5A). This would be compatible with the measured increase in the NADP/NADPH ratio (Fig. S2A). The flow of glucose carbon into the TCA cycle was significantly slower than the labeling of glycolytic intermediates since negligible labeling of TCA cycle intermediates was observed at 5 min (not shown). Despite our observations on the differences in phosphorylation state of PDH, the incorporation of two carbon units into citrate via PDH was not significantly different in the three cell lines (Fig. 5B). Whereas FE of m + 2 citrate reached ~40%, the FE of m + 2 in α-KG (Fig. 5C), malate (Fig. 5D), or aspartate (a surrogate for labeling in oxaloacetate; Fig. 5E) was much lower in all three cell lines (3–15%). In part, this discrepancy could arise if substantial amounts of citrate are exported from the mitochondria to the cytosol for utilization in the ATP citrate lyase reaction that supplies acetyl CoA for lipid biosynthesis. The formation of m + 2 TCA metabolites was highest in the MCU KO cells (FE, 12–15%) indicating increased forward flux in the TCA cycle, which includes the segments containing Ca2+-sensitive isocitrate and α-KG dehydrogenases.

The removal of TCA cycle intermediates for biosynthetic reactions necessitates anaplerotic pathways to replenish these
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molecules. A major anaplerotic reaction involving glucose metabolism is the formation of oxaloacetate from pyruvate by the pyruvate carboxylase (PC) enzyme. This step acting on m + 3 pyruvate would generate m + 3 oxaloacetate which, in addition to being in equilibrium with m + 3 malate (Fig. 5D) and aspartate (Fig. 5E), can also generate m + 3 citrate (Fig. 5B). The formation of m + 3 citrate has been used to monitor PC flux in U−13C-glucose tracing experiments (59). In WT cells, PC flux was small (FE, ~3%) relative to the flux of carbon through PDH (FE, ~40%) (Fig. 5B). However, PC flux was selectively stimulated by ~70% in MCU KO cells (Fig. 5B).

Glutamine is a major substrate feeding carbon into the TCA cycle that can potentially supply both energy and biosynthetic intermediates (60). We therefore analyzed U−13C-glutamine metabolism in the three cell lines using time points of 30 min and 1 h. The sum of label accumulating in several glutamine metabolites (glutamate, α-KG, aspartate, citrate/isocitrate, and cis-aconitate) was taken as a measure of overall glutamine metabolism. This was found to be substantially increased in the MCU KO cells with a much smaller (but significant) increase also noted in the IP3R 3KO cells (Fig. 6A). Metabolism of U−13C-glutamine by the combined actions of glutaminase,
glutamate dehydrogenase, and glutamate transaminases would generate m + 5 labeled α-KG. The “forward” direction of the TCA cycle would generate m + 4 oxaloacetate, and condensation with unlabeled acetyl-CoA would produce m + 4 citrate. Any limitation in the supply of acetyl-CoA as a result of a block at PDH should lower labeling of m + 4 citrate, but this was not observed for either of the KO cell lines (Fig. 6B). Indeed, a small increase was seen in the MCU KO cells. The same pattern of changes was also seen for m + 4 labeled malate (Fig. 6F), indicating no suppression of flux at the Ca²⁺-sensitive α-KG dehydrogenase step. As observed in the glucose labeling data, the oxidative flux through the citrate/α-KG span is increased in the MCU KO cells (Fig. 6E). Carbon entering the TCA cycle as α-KG can also proceed in the “reverse” direction as a result of reductive carboxylation (RC) to generate m + 5 labeled isocitrate, which is in equilibrium with citrate (Fig. 6C). This reverse flux in WT cells was ~20% of the oxidative flux as judged by the relative FEs of m + 4 and m + 5 citrates (Fig. 6, B and C). In IP₃R TKO cells, this ratio of fluxes was increased to ~30%. In MCU KO cells, there was a twofold increase in RC flux, but since the oxidative flux was also increased, the overall partitioning of the two pathways was also ~30%. There is evidence that RC of glutamine can supply acetyl-CoA for fatty acid biosynthesis via mitochondrial citrate export and cleavage by the ATP–citrate lyase reaction (61–63). This reaction acting on m + 5 citrate would generate m + 3 malate (a proxy for m + 3 oxaloacetate). Indeed, the labeling of m + 3 malate in the three cell lines follows the same pattern as m + 5 citrate (Fig. 6G). MCU KO cells showed a significantly increased labeling of m + 2 palmitic acid by acetyl-CoA derived from glutamine metabolism (Fig. 6H).

KO HeLa cells and MCU rescue of HEK293T cells

To determine if the observed phenotypes of the IP₃R TKO and MCU KO are unique to HEK293 cell lines, we examined the effects of these KOs in the HeLa cell line. The IP₃R TKO HeLa cells have been used previously and shown to completely lack histamine-induced cytosolic Ca²⁺ transients (32). CRISPR-mediated gene targeting was used to produce the MCU KO HeLa cells (Fig. 7A). Loss of MCU function in the KO cells was confirmed by the lack of uptake of a pulse of

Figure 7. Characteristics of HeLa cells with IP₃R and MCU KOs and MCU-rescued HEK293T MCU KO cells. A, MCU immunoblotting confirmed loss of MCU in two of four HeLa clones after CRISPR KO. HEK293T WT and MCU-KO cells were used as positive and negative controls, respectively. GAPDH was the loading control. B, cells were permeabilized with saponin, and the clearance of a 50 μM pulse of Ca²⁺ added at 18 s was measured using Fluo-5 as described in the “Experimental procedures” section. A further addition of 1 μM FCCP was made as shown. C, HeLa cells were loaded with Fluo-8 AM as described in the “Experimental procedures” section, and changes to cytosolic Ca²⁺ in response to increasing doses of histamine were measured. D, neutralized PCA lysates of HeLa cells were assayed for lactate. Data are the mean ± SEM (n = 3); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, one-way ANOVA followed by multiple comparison analysis using Dunnett’s test. E and F, phosphorylation status of HeLa PDH and AMPK was measured by immunoblotting. Values are normalized to the total levels of each protein with each point representing the mean of an independent experiment performed in duplicate. G, growth curves for WT, TKO, and MCU-KO HeLa cell lines. Mean ± SEM (n = 3). H, immunoblot for MCU and Flag in MCU-rescue in HEK293T clones. GAPDH was used as a loading control. The lower two panels show phosphorylated and total levels of AMPK. I, Ca²⁺ clearance in permeabilized HEK293T cells was measured as in (D). J, intact HEK293T cells were loaded with Fura-2 as described in the “Experimental procedures” section, and cytosolic Ca²⁺ changes in response to 25 μM carbachol was measured. K, lactate in HEK293T cells was measured in triplicate for three independent experiments as in (D). Statistical evaluation of data in (E–G) and (K) was as in (D). AMPK, AMP-dependent kinase; FCCP, (carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone); HEK293T, human embryonic kidney 293T cell line; IP₃R, inositol trisphosphate receptor; MCU, mitochondrial Ca²⁺ uniporter; PCA, perchloric acid; PDH, pyruvate dehydrogenase.
Ca\(^{2+}\) delivered to permeabilized HeLa cells (Fig. 7B). As noted previously (Fig. 1A), the MCU KO HEK293T cells display a smaller cytosolic Ca\(^{2+}\) response to a saturating dose of Cch. The MCU KO HeLa cells also gave a reduced response to histamine (Fig. 7C) when compared with WT HeLa cells, but the differences were smaller than noted in HEK293 cells. As observed in HEK293T cells, lactate levels were also elevated in MCU KO HeLa cells and were unchanged in IP\(_3\)R TKO cells (Fig. 7D). The smaller effects in HeLa cells may be related to their lesser reliance on glycolysis for ATP production than HEK293 cells (64, 65). The phosphorylation state of PDH (Fig. 7E), AMPK (Fig. 7F), as well as the growth rates of the different HeLa cell lines (Fig. 7G) were all qualitatively similar to the changes observed for the HEK293 cell lines. These data indicate that the observed phenotypes of MCU KO and IP\(_3\)R TKO cells are not unique to the HEK293 cell line.

Another concern of using KO cells is the possibility that the observed phenotypes are due to off-target effects of the knockdown methodology. To determine if this may be the case in the MCU KO HEK293T cells, we sought to determine if stably reintroducing the MCU reverted some of the observed changes. The rescued expression of MCU was confirmed by immunoblotting (Fig. 7H). The restoration of mitochondrial Ca\(^{2+}\) uptake could be demonstrated in Ca\(^{2+}\) clearance assays using permeabilized cells (Fig. 7I). In intact cells, the Cch-induced cytosolic Ca\(^{2+}\) increase was enhanced in the MCU-rescue cells when compared with the MCU KO cells and was approximately 85% of WT cells (Fig. 7J). The increased lactate levels (Fig. 7K) and increased AMPK phosphorylation (Fig. 7H, lower two panels) observed in MCU KO cells were also diminished toward levels observed in WT cells. We conclude that, at least for the parameters measured, the observed phenotype of MCU KO cells is unlikely to be due to off-target effects.

**Forced utilization of the TCA cycle for energy production in the three cell lines**

Constitutive Ca\(^{2+}\)-stimulated mitochondrial oxidative metabolism has been proposed to be particularly important for maintaining the bioenergetics requirements of cancer cells (27, 28). This hypothesis is difficult to test in our KO models because HEK293 cells rely almost entirely on glycolysis to support their energy requirements (66, 67). Galactose metabolism minimizes the energy yield from glycolysis and makes cells increasingly reliant on the TCA cycle and oxidative phosphorylation to supply their energy needs (68). We therefore removed the glucose in DMEM and replaced it with 10 mM galactose. Oligomycin treatment of WT, IP\(_3\)R TKO, or MCU KO cells grown in glucose-containing medium had negligible effects on ATP levels, as expected if oxidative phosphorylation makes a minimal contribution to ATP production (Fig. 8A). By contrast, blocking glycolysis with iodoacetate lowered ATP levels in all three cell lines. Oligomycin markedly reduced ATP levels in all three cell lines incubated in galactose medium, indicating the dominant role of oxidative phosphorylation under these conditions. The ability of the cell lines to maintain their levels of ATP and AMP was followed for different periods after the galactose switch. Over a period of 2 h, the cells had no difficulty in maintaining their ATP and AMP (Fig. 8, B and C). However, more prolonged incubation in galactose led to a progressive decrease in ATP and elevation of AMP in the MCU KO cells but not in the WT or IP\(_3\)R TKO cell lines. When cytotoxicity was measured with a fluorescent dye assay, it was evident that the MCU KO showed evidence of cell death that was significant at 18 h and markedly pronounced at 24 h after galactose incubation (Fig. 8D). There was no evidence of significant cytotoxicity in the WT or IP\(_3\)R TKO cell lines after prolonged incubation in galactose medium. Interestingly, the cytotoxicity observed in the MCU KO cells was strongly suppressed in the MCU-rescue cell line.

**Discussion**

The KO cell models used in this study were intended to compare the chronic metabolic and bioenergetic changes resulting from the loss of either cytosolic Ca\(^{2+}\) signaling (IP\(_3\)R TKO) and/or mitochondrial Ca\(^{2+}\) signaling (MCU KO and IP\(_3\)R TKO). The changes we measured are summarized in the scheme shown in Figure 9. The most prominent changes observed in the MCU KO HEK293T cells were increased glycolysis, glutamine metabolism, and increased growth rate. Presumably, the enhanced consumption of fuels and the increased oxygen consumption of MCU KO cell are needed to meet the demands of increased growth. Varied results have been reported when examining the effects of MCU loss on growth. Although we found an increased growth rate of MCU KO HEK293T and HeLa cells, Hall et al. (42) found HeLa cell proliferation to be decreased not only when MCU levels were diminished with siRNA but also when MCU activity was increased with MICU1 siRNA. Growth was diminished in MCU KO MEFs (47) but unaltered in siRNA-treated MD-MB-231 breast carcinoma cells (42). In agreement with our findings, knockdown of MCU in primary B lymphocytes enhanced proliferation in response to anti-immunoglobulin M (69).

A stimulation of glycolysis has been noted in several mouse models of MCU KO (34, 55) and MCU KO MEFs (47). The usual explanation offered for this effect is that loss of mitochondrial Ca\(^{2+}\) increases the phosphorylation state of PDH, and therefore diminishes its activity, which diverts pyruvate into lactate formation instead of entry into the TCA cycle. Although there are exceptions (39, 53), the loss of MCU is consistently associated with increased p-PDH (34, 50, 55, 56), as also seen in our MCU KO cells (Figs. 3F and 7F). However, there are several reasons for believing that lowered mitochondrial Ca\(^{2+}\) and p-PDH changes are not the underlying reason for the increased glycolysis. First, the mitochondrial Ca\(^{2+}\) content of WT and KO cells is already very low and can be estimated to be much lower than the \(K_d\) of the GEM-GECO probe (i.e., <300 nM). Since the \(K_{a2}\) for PDH activation by Ca\(^{2+}\) in isolated mitochondria is in the range of 300 to 500 nM.
the small changes in baseline Ca\textsuperscript{2+} content are unlikely to have a major impact on levels of p-PDH. Second, only the MCU KO cells show substantially increased PDH phosphorylation, although both MCU KO and IP\textsubscript{3}R TKO cells have comparable mitochondrial Ca\textsuperscript{2+} signals. Previous studies in intact mitochondria have shown that the stimulatory effects of Ca\textsuperscript{2+} on PDH phosphatase are more evident when the activity of PDH kinase is partially inhibited (71). Pyruvate and NADH are both regulators of PDH kinase and their changes in MCU KO cells are in the direction that would act to partially inhibit PDH kinase (72). Since these metabolites do not change in IP\textsubscript{3}R TKO cells, it is possible that the sensitivity of PDH has shifted to a lower range of mitochondrial Ca\textsuperscript{2+} content in MCU KO cells. Finally, the direct measurement of isotope tracer flux of [U-\textsuperscript{13}C] glucose carbon into m + 2 citrate showed no significant differences between the cell lines, indicating that the flow of carbon into the TCA cycle was not diminished despite increased p-PDH. A similar finding was made in a mouse heart MCU KO model using NMR to study the metabolism of [1,2-\textsuperscript{13}C] glucose (73). The increased levels of the PDH substrate pyruvate in the MCU KO cells probably contributes to the maintenance of PDH flux, despite enhanced PDH phosphorylation. If PDH flux is not the critical factor then what else could account for enhanced glycolysis in MCU KO cells? Flux through glycolysis is affected by multiple regulatory mechanisms, but one key factor is the cytosolic NAD\textsuperscript{+}/NADH ratio, which modulates the activity of glyceraldehyde 3-phosphate dehydrogenase. A continuous supply of NAD\textsuperscript{+} to this enzyme is maintained by NADH utilization by the NADH oxidases of the respiratory chain and enzymes such as LDH and malate dehydrogenase in the cytosol. MCU KO cells have an increased NAD\textsuperscript{+}/NADH ratio that is mainly because of a fall in NADH levels (Fig. 2). Both basal respiration and lactate formation are increased in MCU KO cells. Elevated NAD\textsuperscript{+}/NADH ratios with decreased NADH levels has been shown to enhance glycolytic rate in several experimental systems (74–77). An additional factor that may contribute to enhanced glycolysis in MCU KO cells is the activation of AMPK (Fig. 3E), which is known to phosphorylate and stimulate phosphofructokinase-2 (78).

Maintaining the higher growth rate of MCU KO cells requires an increased supply of both energy and biosynthetic
building blocks. It is well established that glutamine metabolism contributes to both functions (79), and enhanced glutamine metabolism often accompanies increased aerobic glycolysis in cancer cells (60). Stimulated glutamine metabolism has been reported in MCU KO fibroblasts (47). Our experiments with [U-13C] glutamine show that MCU KO HEK293T cells have an increased flow of glutamine carbon into the TCA cycle, and the metabolism of α-KG is enhanced in both the oxidative and reductive directions (Fig. 6). The increased flux in the oxidative direction occurs independently of any increase in mitochondrial Ca2+ and may be related to the increased citrate (Fig. S2B) and lowered NAD(P)H (Figs. 2G and S2A), both of which are allosteric modifiers of mitochondrial isocitrate dehydrogenase-3. Increased flux of α-KG in the oxidative pathway is also required to supply the reducing equivalents required for RC (80). RC is commonly observed in the absence of an adequate supply of acetyl-CoA from PDH, as seen, for example, in hypoxia (81). Since PDH flux is not altered in MCU KO cells, the stimulated RC is more likely to be secondary to increased α-KG availability provided by enhanced glutamine metabolism. Interestingly, increased RC also accompanies stimulated glycolysis (82, 83) or high extracellular lactate (84). Both these conditions prevail in MCU KO cells. A further important conclusion from the 13C-tracer studies is the lack of suppression of basal flux through any of the three Ca2+-sensitive TCA cycle dehydrogenases when MCU or IP3Rs are deleted, implying that any small decreases in basal mitochondrial [Ca2+] occurring in the KO cells are not sufficient to impact flux through these enzymes. This conclusion contrasts with the recent findings of Cardenas et al. (29) that constitutive Ca2+ regulation of α-KG dehydrogenase is required to maintain RC in a mutant osteosarcoma cell line with defective oxidative phosphorylation.

The main changes observed in IP3R TKO HEK293 cells were a reduced growth rate, reduced oxygen consumption, and an increased basal rate of autophagy with no changes in glucose or glutamine consumption. Some of these observations are in agreement with previous studies. A lower rate of proliferation and migration in IP3R TKO HEK293 cells has been reported recently (31). A reduced baseline and FCCP-stimulated oxygen consumption was observed in Seahorse assays (85). Acute inhibition or chronic loss of IP3Rs promotes an increase in basal autophagy in several cell types, but different mechanisms for this effect have been proposed (86, 87). In our studies, it is clear that the effects are independent of AMPK activation. Only minor changes in metabolic rewiring could be observed in the 13C-tracer studies, including an increase in anaplerosis of glucose carbon through PC, and an increase in RC of glutamine and palmitate synthesis. All these effects were significantly smaller than observed with MCU KO cells (Figs. 5 and 6). Overall, the data show that the loss of mitochondrial Ca2+ signaling induced by the stable KO of IP3Rs or MCU produce quite distinct phenotypes. We cannot exclude the possibility that some of the changes seen in MCU KO cells may result from the loss of a scaffolding role of the MCU protein itself, rather than the lack of mitochondrial Ca2+ fluxes. Further investigation of the protein–protein interactions of MCU in the mitochondrial matrix and the role of these complexes in regulating the structure/function of mitochondria are required to test this.

Figure 9. Schematic summarizing some of the pathways altered by the chronic loss of IP3Rs and MCU. The acute responses to loss of ER–mitochondrial Ca2+ transport in cancer cells as documented in the literature (29, 30, 94) are shown above the dotted line. The adaptive chronic changes measured in the present study are shown below the dotted line. Upregulated or downregulated parameters are indicated by arrows colored red for IP3R TKO and blue for MCU KO. The absence of an arrow indicates no change or minimal change in that parameter. Unlike acute effects, the chronic loss of ER–mitochondrial Ca2+ signaling maintains ATP levels and avoids bioenergetic failure. ER, endoplasmic reticulum; IP3R, inositol trisphosphate receptor; MCU, mitochondrial Ca2+ uniporter.
Metabolic adaptation to loss of calcium signaling

Calcium is viewed as a universal second messenger regulating a wide variety of biological processes (1). Global and conditional mouse KOs of IP3R isoforms have supported a biologically important role for intracellular Ca2+ signals (15). Yet, it is also apparent that several cancer cells (e.g., HEK293, HeLa, and DT40 lymphocytes) can survive the loss of all three IP3R isoforms. Several evolutionary lineages either lack recognizable IP3Rs (89, 90) or have independently lost IP3Rs while retaining MCU homologs (91, 92). Thus, the phenotype of cells without Ca2+ signaling may utilize ancestral Ca2+-independent signaling mechanisms and/or additional transcriptional rewiring resulting from disruption of IP3R genes. Future RNA-Seq studies could provide useful information on the molecular mechanisms underlying the adaptive responses of growth and metabolism seen after loss of IP3Rs and MCU.

Targeting Ca2+ signaling with drugs that block ER–mitochondrial communication is being explored as a strategy for the treatment of various diseases where there is aberrant translocation of Ca2+ into the mitochondria (93–95). Chronic use of these drugs may induce metabolic and bioenergetic adaptations similar to those seen in the present study. Understanding these mechanisms would aid in optimizing the therapeutic benefits of blocking Ca2+ signaling in disease states.

**Experimental procedures**

**Cell lines and culture**

All cells were cultured at 37 °C and 5% CO2 in DMEM supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 0.25 μg/ml amphotericin B. HEK293 IP3R TKO cells and HeLa IP3R TKO cells were made as described previously (30, 32). Most of the experiments in this study used HEK293T MCU KO cells made by TALEN knockdown that were obtained from the laboratory of Dr Vamsi Mootha (48). The appropriate controls for the IP3R TKO and MCU KO cells are WT HEK293 cells and HEK293T cells, respectively. We have compared several parameters investigated in the present study and did not find them to be significantly different in the controls (Figs. 1A and 2A and S1). Therefore, the main figures show only the WT HEK293 cells as the controls for both IP3R TKO and MCU KO cells.

**Ca2+ measurements**

Trypsin-released cells were seeded on a black, clear-bottom, and 96-well plate at a density of 5 × 10⁵ cells/well and incubated at 37 °C and 5% CO2 for 24 h. Media were aspirated from all wells of the plate and replaced with 190 μl of Ca2+-assay buffer (Hank’s balanced salt solution containing MgCl2 [1 mM], CaCl2 [2.3 mM], and 20 mM Hepes [pH 7.2]) supplemented with 5 μM Fluo-AM, 500 μM Brilliant Black, and 100 μM sulfipyrazone. The plate was maintained in the incubator for 45 min. Changes in cytosolic Ca2+ were monitored using a FlexStation II plate reader in fluorescence mode at 37 °C. Measurements were made using excitation and emission wavelengths of 485 and 525 nm, respectively. The additions of Cch, FCCP, oligomycin, and ionomycin were
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made by utilizing the automated fluidics system of the hardware. In some experiments, cells were loaded as aforementioned with 1 μM Fura2-AM, and Ca2+ was measured ratiometrically using excitation and emission wavelengths of 340/380 and 510 nm, respectively. Ca2+ fluxes in permeabilized cells were measured after harvesting cells grown in 100 mm plates with trypsin. The cells were centrifuged (1000g; 2 min), washed in Ca2+/Mg2+-free Hank’s balanced salt solution and then resuspended in 600 μl of dye buffer (2 μM Fluo-5N, 120 mM KCl, 1 mM MgCl2, 25 mM Hepes [pH 7.2], 5 mM sucinate, 2 mM Tris[OH], 4 μM rotenone, and 1 μM thapsigargin), and permeabilized using saponin (10 μg). Permeabilization was verified by trypan blue staining. Aliquots (90 μl) were transferred to wells of a black transparent bottom 96-well plate. The plate was maintained at 37 °C, and measurements were made using excitation and emission wavelengths of 491 and 516 nm, respectively.

For imaging experiments, cells were preincubated in a serum-free extracellular medium (121 mM NaCl, 5 mM NaHCO3, 10 mM Na–Hepes, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, and 10 mM glucose [pH 7.4]) containing 2% bovine serum albumin (BSA). For measurements of [Ca2+]i, cells were loaded with 2 μM Cal520-AM (AAT Bioquest) together with 0.003% pluronic F127 and 100 μM sulfipyrazine for 30 min at 30 °C. At the end of the preincubation or dye-loading period, the cells were washed into fresh extracellular medium containing 0.25% BSA and 0.25% trypsin/EDTA and centrifuged (1000 g; 5 min). The supernatant was resuspended in 1 ml PBS, split into two equal aliquots, and recentrifuged. For NAD+/NADP+ assay, the cell pellet was resuspended with 100 μl of 1 M KOH, heated at 65 °C for 30 min, and briefly sonicated (10 s). All samples were stored at −80 °C prior to assay. NAD+ and NADH were measured by an enzymatic cycling assay using alcohol dehydrogenase (99). NADP+ and NADPH were measured by an enzymatic cycling assay using glucose-6-phosphate dehydrogenase (100). ATP was assayed in neutralized PCA extracts using a luciferase method (101). AMP was assayed using the AMP-glo kit (Promega) following the manufacturer’s instructions.

Growth curves

About 2.5 × 105 HEK293, HEK293T, or HeLa cell lines were plated on 100 mm plates. At the indicated times, plates were trypsinized for 4 min and resuspended in 10 ml DMEM. The cell density was counted in triplicate using a hemocytometer.

Metabolite assays

Glucose consumption was measured after a 1 h period in which the cells were incubated in glucose-free medium. About 5 mM glucose was then added, and aliquots of the media (250 μl) were removed for glucose assay at the indicated times. A previously described colorimetric method using glucose oxidase was modified for use on a plate reader as described (96). Lactate was measured in neutralized perchloric acid (PCA) lysates using LDH as previously described (97). Pyruvate was measured with pyruvate oxidase and Amplex red (98). Nicotinamide nucleotides were measured using enzymatic cycling assays. Cells grown on 10 cm2 plates were released with 0.25% trypsin/EDTA and centrifuged (100g; 5 min). The pellets were resuspended in 1 ml PBS, split into two equal aliquots, and recentrifuged. For NAD+/NADP+ assay, the cell pellet was resuspended with 100 μl 0.6 M PCA. For NADH/ NADPH assay, the cell pellet was resuspended in 100 μl of 1 M KOH, heated at 65 °C for 30 min, and briefly sonicated (10 s). All samples were stored at −80 °C prior to assay. NAD+ and NADH were measured by an enzymatic cycling assay using alcohol dehydrogenase (99). NADP+ and NADPH were measured by an enzymatic cycling assay using glucose-6-phosphate dehydrogenase (100). ATP was assayed in neutralized PCA extracts using a luciferase method (101). AMP was assayed using the AMP-glo kit (Promega) following the manufacturer’s instructions.

SDS-PAGE/Western blotting

To prepare lysates, cells incubated in DMEM were washed in PBS and lysed in a medium (0.250 ml) containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.8), 150 mM NaCl, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM NaF, and a 1× dilution of a complete protease inhibitor mixture (Roche Diagnostics). The lysates were centrifuged at 12,000g for 10 min. The supernatants were denatured in SDS sample buffer. Lysates were boiled at 100 °C for 5 min and then stored at −20 °C until use. Unless otherwise noted, 40 μg of protein were run at 100 V for 90 min on 10% polyacrylamide gels and transferred at 100 V for 60 min onto nitrocellulose membranes. Polyvinylidene difluoride membranes were used in the specific case of LC3 immunoblotting. Membranes were blocked in Tris-buffered saline with Tween-10 supplemented with 5% BSA for 1 h at room temperature. Following 3 × 15 min washes in Tris-buffered saline with Tween-10, the primary antibody was added for 16 h at 4 °C. All the antibodies were used at a dilution of 1:1000 and were obtained from Cell Signaling. Membranes were developed using ECL reagent (Thermo Fisher Scientific). When necessary, membranes were stripped using a buffer containing SDS (2%; w/v), Tris (62.5 mM; pH 6.8), and β-mercaptoethanol (100 mM).

Oxygen consumption

Cells were seeded at a density of 2 × 105 and were grown on 60 mm plates for 5 days. Trypsinized cells (106) resuspended in DMEM were introduced into the chamber of an O2 electrode (Oroboros Instruments GmbH). Baseline rates of O2 consumption and the rates after sequential addition of 5 μM oligomycin, 10 μM FCCP, and 1 μM antimycin A were measured.

Labeled isotope tracing

Cells were seeded on 60 mm plates with 2 × 105 cells in DMEM and grown for 5 days. To label with glutamine, the DMEM, containing all other components except glutamine, was supplemented with 4 mM 1-[U–13C] glutamine. The plates were labeled in triplicate with 1 ml of medium for
30 and 60 min. To label with glucose, the DMEM, containing all other components except glucose, was supplemented with 5 mM D-[U-13C] glucose. The plates were labeled in triplicate with 1 ml medium for 5 and 60 min. At the indicated times, the plates were aspirated and rapidly washed in ice-cold PBS. Metabolites were extracted by rocking in 0.5 ml buffer containing 5:3:2 ratios of methanol, acetonitrile, and water for 10 min at 4 °C. The solvent was removed, centrifuged at 12,000g for 10 min, and stored at ~80 °C prior to analysis. LC–MS analysis was performed on a Q Exactive HybridQuadrupole-Orbitrap HF-X MS (Thermo Fisher Scientific) equipped with a HESI II probe and coupled to a Vanquish Horizon UHPLC system (Thermo Fisher Scientific). Conditions for chromatography, mass spectroscopy, identification, and quantitation of metabolites were as detailed in the study by Casciano et al. (102).

**CRISPR–Cas9 MCU KO and rescue**

MCU KO clones of HeLa cells were obtained using Cas9/guide RNA ribonucleoprotein complexes (103). Two CRISPR RNA sequences were used: GAUCGCUUCGCAGAA UUGUUUAAGCUAUGCU; UGAACUCAGCGCUUCA CGCGUUUAGACGAUUGCU. Guide RNA complexes were formed by annealing the CRISPR RNA with transactivating RNA as described by Jacobi et al. (104). The complexes were combined with recombinant Cas9 nuclease and transfected into HeLa cells with Lipofectamine 3000. RNAs and Cas9 were purchased from Integrated DNA Technologies. After 1 week of growth, the cells were subcloned by limiting dilution into 96-well plates. Individual clones were expanded into 35 mm plates and screened for MCU expression by immunoblotting. MCU rescue in MCU KO HEK293T cells was carried out by transfecting a plasmid encoding a C-terminal FLAG-tagged human MCU (Addgene; catalog no. 50054) using Lipofectamine 3000 and selecting for stable transfectants using hygromycin (20 μg/ml).

**Data collection and analysis**

Unless otherwise noted, the data are expressed as means ± SEM of three independent experiments, each performed in technical triplicate. GraphPad Prism (GraphPad Software, Inc) was used to generate plots and perform statistical analysis. A complete list of statistical analysis of the figures can be found in Table S1.

**Data availability**

All data generated or analysed during this study are included in this published article (and its supplementary information files).

**Supporting information**—This article contains supporting information.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: AMPK, AMP-dependent kinase; BSA, bovine serum albumin; Cch, carbachol; DMEM, Dulbecco’s modified Eagle’s medium; ER, endoplasmic reticulum; FCCP, (carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone); FE, fractional enrichment; HEK293, human embryonic kidney 293 cell line; IP₃R, inositol trisphosphate receptor; α-KG, α-ketoglutarate; LC3, microtubule-associated protein light chain 3; LDH, lactate dehydrogenase; MCU, mitochondrial Ca²⁺ uniporter; MEF, mouse embryonic fibroblast; OCR, oxygen consumption rate; PC, pyruvate carboxylase; PCA, perchloric acid; PDH, pyruvate dehydrogenase; p-PDH, phosphorylated PDH; RC, reductive carboxylation; TCA, tricarboxylic acid; TKO, triple KO.

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Metabolic adaptation to loss of calcium signaling

Metabolic adaptation to loss of calcium signaling is a critical process that affects cellular function and is essential for maintaining homeostasis. The loss of calcium signaling can significantly impact cellular processes, including metabolism, autophagy, and cell cycle progression. This section highlights the role of calcium signaling in metabolic adaptation and its implications for various cellular processes.

1. **Introduction**

Metabolism is a complex process that involves the conversion of nutrients into energy and other essential molecules. Calcium signaling plays a crucial role in regulating cellular metabolism by modulating various metabolic pathways. The loss of calcium signaling can alter the metabolic profile of cells, leading to metabolic adaptation.

2. **Role of Calcium Signaling in Metabolism**

Calcium signaling is a fundamental process that regulates numerous cellular functions, including metabolism. Calcium signaling is involved in the regulation of metabolic pathways, such as glycolysis, lipid metabolism, and autophagy. The loss of calcium signaling can disrupt these processes, leading to metabolic adaptation.

3. **Metabolic Adaptation**

Metabolic adaptation is a physiological process that allows cells to adjust their metabolic pathways in response to changes in the cellular environment. This adaptation helps cells maintain optimal energy production and growth. The loss of calcium signaling can promote metabolic adaptation by altering the expression of metabolic genes and enzymes.

4. **Calcium Signaling and Metabolic Pathways**

Calcium signaling is involved in the regulation of various metabolic pathways, including glycolysis, lipid metabolism, and autophagy. The loss of calcium signaling can alter the expression of genes and enzymes involved in these pathways, leading to metabolic adaptation.

5. **Implications for Cellular Function**

The loss of calcium signaling can have significant implications for cellular function and survival. Metabolic adaptation can help cells cope with changes in the cellular environment, but it can also lead to metabolic diseases if the adaptation process is not properly regulated.

6. **Conclusion**

In conclusion, calcium signaling plays a crucial role in regulating cellular metabolism. The loss of calcium signaling can promote metabolic adaptation, which can have significant implications for cellular function and survival. Understanding the mechanisms of metabolic adaptation in response to the loss of calcium signaling is essential for developing new therapeutic strategies to treat metabolic diseases.
Metabolic adaptation to loss of calcium signaling

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