CALCIUM SIGNALING

Loss of MCU prevents mitochondrial fusion in G1-S phase and blocks cell cycle progression and proliferation

Olha M. Koval1, Emily K. Nguyen1, Velarchana Santhana1, Trevor P. Fidler2,3, Sara C. Sebag1, Tyler P. Rasmussen1, Dylan J. Mittauer1, Stefan Strack4, Prabhat C. Goswami5, E. Dale Abel1,2, Isabella M. Grumbach1,2,5,6*

The role of the mitochondrial Ca2+ uniporter (MCU) in physiologic cell proliferation remains to be defined. Here, we demonstrated that the MCU was required to match mitochondrial function to metabolic demands during the cell cycle. During the G1-S transition (the cycle phase with the highest mitochondrial ATP output), mitochondrial fusion, oxygen consumption, and Ca2+ uptake increased in wild-type cells but not in cells lacking MCU. In proliferating wild-type control cells, the addition of the growth factors promoted the activation of the Ca2+/calmodulin-dependent kinase II (CaMKII) and the phosphorylation of the mitochondrial fission factor Drp1 at Ser616. The lack of the MCU was associated with baseline activation of CaMKII, mitochondrial fragmentation due to increased Drp1 activation, and impaired mitochondrial respiration and glycolysis. The mitochondrial fission/fusion ratio and proliferation in MCU-deficient cells recovered after MCU restoration or inhibition of mitochondrial fragmentation or of CaMKII in the cytosol. Our data highlight a key function for the MCU in mitochondrial adaptation to the metabolic demands during cell cycle progression. Cytosolic CaMKII and the MCU participate in a regulatory circuit, whereby mitochondrial Ca2+ uptake affects cell proliferation through Drp1.

INTRODUCTION

The mitochondrial Ca2+ uniporter (MCU) is a highly conserved protein of the inner mitochondrial membrane that mediates the electrophoretic Ca2+ uptake into the mitochondrial matrix (1, 2). MCU-dependent Ca2+ uptake increases tricarboxylic acid cycle activity by activating Ca2+-dependent hydrogenases (3). Accordingly, MCU deficiency in cardiac muscle prevents the adaptation of energy supply to states of maximal demand (4). However, the role of MCU in less metabolically demanding physiologic processes such as cell proliferation remains unclear (5).

During the cell cycle, fluctuations in intracellular Ca2+ levels and transients occur (6) and drive the periodic activation of Ca2+-sensitive regulators of proliferation such as Ca2+/calmodulin (CaM), Ca2+/CaM-dependent kinases (CaMK), and the phosphatase calcineurin (7, 8). Inhibition of mitochondrial Ca2+ uptake in proliferating smooth muscle cells disturbs cytosolic Ca2+ transients (9, 10). In a pancreatic β cell line, increased mitochondrial Ca2+ concentration in S and G2-M phases is associated with dynamic changes in cytosolic Ca2+ (11). Thus, because mitochondrial Ca2+ uptake contributes to the fluctuations in Ca2+ compartmentalization throughout the cell cycle (11), MCU may represent a fundamental but underappreciated regulator of cell cycle progression. MCU−/− mice at all ages have relatively lower body and organ weight compared to their wild-type (WT) littermates, providing indirect evidence for a role of MCU in cell proliferation (12).

Dynamic changes in mitochondrial morphology promote early cell cycle progression (13), with mitochondria converting from a highly fragmented state in G0 to a hyperfused network at G1-S transition. This event correlates with the high mitochondrial adenosine 5’-triphosphate (ATP) output in cells that are otherwise predominantly dependent on glycolysis (13). The Ca2+-dependent proliferation effectors CaMKI, CaMKII and cyclin-dependent kinase 1 (CDK1), and calcineurin regulate mitochondrial fission mediated by the guanosine triphosphatase (GTPase) dynamin related protein 1 (Drp1) (14–18). Altered cytosolic Ca2+ levels caused by decreased Ca2+ mitochondrial uptake are thus expected to promote fission mediated by Drp1, providing a potential mechanistic link for MCU-dependent proliferation. Here, we hypothesized that cell cycle progression requires coordination of mitochondrial and cytosolic Ca2+ levels and that MCU deficiency impairs cell proliferation by altering cytoplasmic Ca2+ transients, mitochondrial dynamics due to altered Drp1 phosphorylation, and mitochondrial energetics.

RESULTS

MCU deficiency decreases cell proliferation in vivo and in vitro

Wound closure after cutaneous punch biopsy was significantly delayed in MCU−/− mice, with a 60% reduction in wound size compared to 85% in littermate WT mice on day 10 after injury (Fig. 1, A and B). We also determined the area and number of cell nuclei in the aorta, an organ with limited postnatal proliferation, to test for developmental differences (Fig. 1, C to E). Hematoxylin and eosin (H&E) staining of aortic cross sections from 12-week-old mice demonstrated a significant reduction in the area and number of nuclei in the medial layer in MCU−/− mice compared to WT littermates. The ratio of cells to area was similar in both genotypes, suggesting that the difference in

1Abbud Cardiovascular Research Center, Division of Cardiovascular Medicine, Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA. 2Fraternal Order of Eagles Diabetes Research Center and Division of Endocrinology and Metabolism, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA. 3Department of Molecular Medicine, Columbia University Medical Center, New York, NY 10032, USA. 4Department of Pharmacology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA. 5Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, University of Iowa, Iowa City, IA 52242, USA. 6Veterans Affairs Healthcare System, Iowa City, IA 52246, USA. *Corresponding author. Email: isabella-grumbach@uiowa.edu
these results implicate MCU as a regulator of cell proliferation. Moreover, as reported previously (12), male and female MCU−/− spleen, heart, and kidney were significantly reduced in days 18, 21, and 33 (fig. S1, C to E). Although the weights of the were significantly smaller than their WT littermates on postnatal

MCU−/− in WT and


Fig. 1. Deletion of MCU reduces cell proliferation in vivo and in vitro. (A) Interscapular wounds after skin punch in WT and MCU−/− mice. (B) Quantification of wound area. n = 8 mice per genotype. (C) H&E-stained cross sections of the descending aorta. Scale bars, 100 μm. (D) Quantification of aortic medial cross-sectional areas. n = 12 mice per genotype. (E) Quantification of cell nuclei in the aortic media. (F) Ratio of nuclei per area, derived from (E) and (D). (G) Cell counts for cultured VSMCs transfected with MCU or scrambled siRNA in growth medium with PDGF (20 ng/ml). (H) Cell counts for VSMCs treated with the MCU inhibitor RU360 (100 nM) and/or PDGF. n = 8 independent experiments. (I) Cell counts for skin fibroblasts from MCU−/− or WT mice at 72 hours in growth medium with PDGF (20 ng/ml). n = 9 independent experiments. (J) TUNEL staining in VSMCs transfected with MCU or scrambled siRNA and treated with PDGF (20 ng/ml) for 48 hours. n = 6 independent experiments. *P < 0.05, **P < 0.001 by two-way repeated measures analysis of variance (ANOVA) (B), Mann-Whitney U test (D, F, and I), two-tailed t test (E), one-way ANOVA at 48 hours (G and H), and Kruskal-Wallis test (J).

size was at least partially driven by reduced cell numbers (Fig. 1F). To assess postnatal physiological organ regeneration, we labeled proliferating hepatocytes by proliferating cell nuclear antigen (PCNA) immunostaining. Significantly fewer PCNA-positive cells were detected in liver sections from MCU−/− mice (fig. S1, A and B). Together, these results implicate MCU as a regulator of cell proliferation. Moreover, as reported previously (12), male and female MCU−/− mice were significantly smaller than their WT littermates on postnatal days 18, 21, and 33 (fig. S1, C to E). Although the weights of the spleen, heart, and kidney were significantly reduced in MCU−/− mice, the organ weight adjusted to total body weight was not different between genotypes, supporting a general effect of MCU deletion on organ development and growth (fig. S1, F to H).

Acute silencing of MCU by small interfering RNA (siRNA) or pharmacological inhibition with RU360 significantly reduced the number of WT vascular smooth muscle cells (VSMCs) in vitro at 72 hours after treatment with platelet-derived growth factor (PDGF; Fig. 1, G and H), findings that were confirmed by cell counts of cultured skin fibroblasts from MCU−/− and WT mice (Fig. 1I). MCU inhibition reduces apoptotic cell death, making it unlikely that the Ca2+ adenosine triphosphatase (SERCA) inhibitor thapsigargin. The cytoplasmic Ca2+ transients in MCU−/− VSMCs were prolonged compared to WT (Fig. 2, E and F). The Fura-2 AM signal at baseline was similar between genotypes (Fig. 2G). Last, we recorded the cytoplasmic Ca2+ levels after PDGF administration over 50 min (3000 s; Fig. 2H). After the initial treatment, the cytoplasmic Ca2+ levels returned to baseline in WT but not in MCU−/− VSMCs in which the repeated addition of PDGF further increased cytoplasmic Ca2+ levels. These data demonstrate that MCU inhibition prolongs cytoplasmic Ca2+ transients after PDGF treatment in VSMCs.

MCU inhibition prolongs cytosolic Ca2+ transients

MCU knockdown by siRNA (fig. S2, A and B) decreased PDGF-induced mitochondrial Ca2+ transients, particularly the peak amplitude and the area under the curve (AUC), as measured using the mitochondrial Ca2+ indicator mtPericam (fig. S2, C to E). These findings were recapitulated with MCU deletion or inhibition with RU360 (fig. S2, F to H). The effects of MCU deficiency on cytoplasmic Ca2+ levels were evaluated by Fura-2 acetoxymethyl ester (AM) imaging of proliferating VSMCs. Inhibition or knockout of MCU significantly increased the peak amplitude and AUC of cytosolic Ca2+ transients after PDGF treatment (Fig. 2, A to C), suggesting higher peak concentrations and impaired clearance. The up-take of mitochondrial Ca2+ and its release after application of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) were reduced in MCU−/− skin fibroblasts (Fig. 2D). To determine endoplasmic reticulum (ER) Ca2+ content, we treated WT and MCU−/− skin fibroblasts with the sarcoplasmic reticulum

MCU is required to adapt mitochondrial ATP production to energy demands during the cell cycle

We next sought to determine how MCU deletion affects cell cycle progression and analyzed WT or MCU−/− fibroblasts in growth arrest and at 16 and 24 hours after release from arrest. At 16 hours, 41% of WT fibroblasts had entered S phase compared to 18% of MCU−/− cells (Fig. 3, A and B), and cyclin D and E protein abundance significantly increased in WT but not in MCU−/− fibroblasts (Fig. 3, C to E). To corroborate that MCU deletion impaired S-phase entry, cells were detected differences in cell numbers result from increased apoptosis (12, 19). This notion was confirmed by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining of VSMCs with MCU knockdown (Fig. 1). Together, these data support that MCU deficiency reduces cell proliferation in fibroblasts and dedifferentiated smooth muscle cells.
arrested at the G1-S transition by incubation with aphidicolin, an inhibitor of DNA polymerases. At 5 hours after removal of aphidicolin, significantly more WT than MCU−/− fibroblasts had entered S phase (Fig. 3, F and G). We also confirmed these findings by assessing the cell cycle progression in WT and MCU−/− VSMCs (Fig. 3, A and B). Mitochondrial fusion occurs at the G1-S transition (13), and progression to S phase correlates with greater mitochondrial ATP production (11). As anticipated, mitochondria were predominantly fragmented in growth-arrested WT and MCU−/− fibroblasts (Fig. 3, H and I). Whereas mitochondria in WT fibroblasts elongated within 16 hours of exposure to growth factors, mitochondria in MCU−/− fibroblasts remained mostly fragmented.

Next, we investigated how MCU deletion affects cytosolic Ca2+ levels and mitochondrial respiration. Cytosolic Ca2+ concentrations were significantly increased in WT fibroblasts at 16 hours after release from growth arrest but not in MCU−/− fibroblasts (Fig. 4A). Moreover, WT cells showed that mitochondrial Ca2+ uptake increased 16 hours after release from growth arrest in parallel with a rise in oxygen consumption rate (OCR), but these changes did not occur in MCU−/− cells (Fig. 4, B and C). Because mitochondrial fusion promotes mitochondrial Ca2+ uptake that fuels oxidative phosphorylation and mitochondrial ATP production (13), our data suggest that MCU is required to adapt mitochondrial ATP production to energy demands during the cell cycle. To test whether the expression of proteins in the MCU complex changes during the cell cycle, we performed immunoblot in mitochondria of WT and MCU−/− fibroblasts at 16 and 24 hours after release from cell cycle arrest (Fig. 4D). The protein levels of the regulatory subunits mitochondrial calcium uniporter (MCU) and essential MCU regulator (EMRE) at 16 hours were reduced compared to those at 0 and 24 hours, and the levels of Na+/Ca2+/Li+ exchanger (NCLX) and mitochondrial calcium uptake 1 protein (MICU1) at 16 hours were lower than those at 0 hours (Fig. 4, F to H). MCU deletion led to loss of EMRE, reduced levels of NCLX, and attenuated the changes throughout the cell cycle (Fig. 4D). These data suggest that the dynamic regulation of the MCU complex during the cell cycle leads to increased mitochondrial Ca2+ uptake in S phase in parallel to mitochondrial fusion and increased mitochondrial respiration.

**MCU affects mitochondrial dynamics**

Mitochondrial fission and fusion are controlled by GTPases, including Drp1, a key regulator of mitochondrial fission at the outer mitochondrial membrane (20). Drp1 is regulated by phosphorylation, which is partially controlled by Ca2+-dependent signaling (14–16). The phosphorylation of Drp1 at Ser618 promotes mitochondrial fragmentation (18). Because we detected mitochondrial fission in MCU-inhibited cells, we hypothesized that these findings were due to increased Drp1 phosphorylation. As anticipated, acute PDGF treatment induced Drp1 phosphorylation at Ser618 in proliferating WT VSMCs (Fig. 5, A and B). However, VSMCs with MCU knockdown displayed high baseline Drp1 phosphorylation that did not further increase with PDGF. Accordingly, mitochondrial fission was induced by PDGF in proliferating control cells but not in VSMCs with MCU knockdown that displayed fragmented mitochondria at baseline (Fig. 5, A and B). These findings were recapitulated in vivo. Baseline Drp1 phosphorylation was significantly higher in the heart and aorta of MCU−/− mice (Fig. 5, C and D). Once activated, Drp1 associates with the outer mitochondrial membrane (16). Accordingly, at baseline, significantly more Drp1 associated with mitochondria in MCU knockout cells than in WT cells (Fig. 5, E and F). In response to PDGF, colocalization of Drp1 with mitochondria increased in WT cells but not in MCU-deficient cells.

**Drp1 and its phosphorylation by CaMKII are required to inhibit proliferation in MCU-deficient cells**

To dissect whether Drp1 regulates the proliferation defect in MCU−/− cells, we used Drp1−/− fibroblasts with MCU knockdown or overexpression. Drp1 deletion alone significantly reduced the number of proliferating cells; however, gain or loss of MCU expression did not affect cell numbers beyond those observed after Drp1 knockout (Fig. 5, G to I). These data demonstrate that the presence of Drp1 is required for the effects of MCU on cell proliferation. Several kinases

---

**Fig. 2. MCU activity is required for cytosolic Ca2+ clearance.** (A) PDGF-evoked Ca2+ transients in WT and MCU−/− VSMCs and WT VSMCs pretreated with 100 nM RU360 for 16 hours (arrow: addition of 10 nM PDGF). (B) AUC for (A). n = 5 independent experiments. (C) Peak amplitude for (A). n = 5 independent experiments with 8, 7, and 6 biological replicates for WT, MCU−/−, and RU360 treatments, respectively. (D) Mitochondrial Ca2+ uptake by Calcium Green-SN assay in WT and MCU−/− skin fibroblasts. Treatment with digitonin (Dig; 0.005%), Ca2+ (1 mM), and FCCP (25 μM). n = 2 independent experiments. AU, arbitrary units. (E) Thapsigargin (Thap)–induced Ca2+ transients in WT and MCU−/− VSMCs and WT VSMCs pretreated with 100 nM RU360 (arrow: addition of 1 μM thapsigargin). (F) AUC in (E). n = 5 independent experiments with 6, 7, and 5 replicates for WT, MCU−/−, and RU360 treatments, respectively. (G) Quantification of baseline Fura signal in untreated WT VSMCs, WT VSMCs transfected with MCU siRNA, and MCU−/− VSMCs. n = 5 independent experiments with 12, 9, and 8 replicates for WT, MCU−/−, and WT VSMCs transfected with MCU siRNA, respectively. *P < 0.05, **P < 0.01 compared to WT untreated by Kruskal-Willis test (B) and one-way ANOVA (C, F, and G). (H) Cytosolic Ca2+ levels by Fura recording in WT and MCU−/− VSMCs after PDGF treatment (arrows) recorded over 3000 s. n = 4 independent experiments.
including protein kinase C (PKC), CDK1, and CaMKII promote the phosphorylation of Drp1 at Ser616 (14–16, 21). A screen for PKC phosphorylation targets showed no increased PKC activity in tissue samples of MCU−/− mice (fig. S5, A and B). However, increased CaMKII activation by autophosphorylation was detected in MCU−/− VSMCs at baseline (Fig. 6, A and B), which did not further increase after PDGF treatment. In contrast, in WT VSMCs, PDGF induced a robust activation of CaMKII. In parallel to the association of Drp1 with mitochondria upon PDGF treatment in WT cells, more CaMKII was detected in mitochondrial fractions at 30 min after addition of PDGF (fig. S6, A to C). Consistent with in vitro data, CaMKII activation was higher in the skin and aorta of MCU−/− mice than in those from WT mice (Fig. 6, C and D). Treatment of WT VSMCs with the CaMKII inhibitor KN93 significantly decreased Drp1 phosphorylation in response to PDGF, suggesting a link between CaMKII activity and Drp1 phosphorylation (Fig. 6, E and F). Overexpression of the dominant negative CaMKII T287A mutant, which is missing a functional autophosphorylation site, promoted mitochondrial fusion in cells of both genotypes at baseline and restored PDGF-induced fission in MCU−/− VSMCs, demonstrating that alterations in mitochondrial fission are due to CaMKII-driven Drp1 phosphorylation (Fig. 6, G and H). Similarly, CaMKII inhibition by adenoviral overexpression of the potent and specific CaMKII inhibitor peptide CaMKII N (22) normalized Drp1 phosphorylation at baseline and restored PDGF-induced Drp1 phosphorylation and mitochondrial fission only in MCU−/− cells (Fig. 6, I to L).

**MCU is necessary to maintain mitochondrial reserve capacity in VSMCs**

Mitochondrial Ca2+ uptake through MCU fuels electron transport chain protein activity and is necessary to maintain mitochondrial reserve capacity in cardiac myocytes (12). We tested whether this is also true in proliferating VSMCs. First, we established that baseline and reserve respiration and extracellular acidification rate (ECAR) were impaired in MCU−/− cells under growth conditions (Fig. 7, A to C). CaMKII inhibition restored respiration and lactate production in MCU−/−

---

**Fig. 3.** MCU−/− skin fibroblasts have blunted cell cycle progression from G1-S phase. (A) Representative fluorescence-activated cell sorting analysis for DNA content in synchronized/growth-arrested WT and MCU−/− skin fibroblasts at 0, 16, and 24 hours after release from arrest with 10% fetal bovine serum (FBS) + PDGF (10 ng/ml). (B) Cell cycle phase distribution of fibroblasts from (A), n = 6 independent experiments with 11 and 12 replicates for WT and MCU−/−, respectively. (C) Immunoblots for cyclin D and cyclin E in synchronized/growth-arrested skin fibroblasts (0 hours) and at 16 hours after 10% FBS + PDGF treatment (10 ng/ml). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (D) Quantification of cyclin D levels as in (C), n = 7 independent immunoblots. (E) Quantification of cyclin E levels as in (C). (F) Progression to S phase after release from G1 arrest with aphidicolin (Aph), n = 4 independent experiments with a total of 7 and 8 replicates for WT and MCU−/−, respectively. (H) Confocal microscopy images of skin fibroblasts at baseline and 16 hours after PDGF-induced cell cycle progression [mitochondrial-targeted green fluorescent protein (mtGFP); green], Scale bars, 20 μm (larger image) and 5 μm (inset). (I) Mitochondrial form factor in skin fibroblasts in (H), n = 5 independent experiments, with the number of cells analyzed indicated in bars. *P < 0.05, **P < 0.01, ****P < 0.001 by Kruskal-Wallis test.
S phase and respiration necessary for cell cycle

Mitochondrial respiration in MCU−/−

36

23

tion of Drp1 with Fis1 ( ). P110 delivery improved mitochondrial

Drp1 inhibitor peptide P110. P110 specifically inhibits the interac­

tion of mitochondrial fragmentation, we blocked mitochondrial fission with the
deletion on mitochondrial respiration was due to exaggerated mito­
tochondrial Ca2+ uptake (fig. S9A), decreased baseline Drp1

phosphorylation and mitochondrial fission, and restored the response

to PDGF (fig. S9, B to D). MCU overexpression improved mito­

chondrial respiration and ECAR in VSMCs while decreasing proliferation in WT cells (Fig. 7H).

Inhibition of PKC by Go6083 had no effect on respiration in both
genotypes (fig. S7, A and B). Because we used cells with a constitu­
tive MCU deletion, these findings could be due to adaptation to con­
stitutive MCU deletion as opposed to MCU deficiency. Thus, we also

measured mitochondrial bioenergetics in VSMCs with acute MCU

knockdown, which confirmed the findings in MCU−/−VSMCs (fig. S8,

A to F). Moreover, overexpression of MCU in MCU−/−cells increased mitochondrial Ca2+ uptake (fig. S9A), decreased baseline Drp1

phosphorylation and mitochondrial fission, and restored the response to

PDGF (fig. S9, B to D). MCU overexpression improved mito­

chondrial respiration and ECAR in MCU−/−cells but not in WT

cells (fig. S9, E and F). To test whether the inhibitory effect of MCU
deletion on mitochondrial respiration was due to exaggerated mito­
tochondrial fragmentation, we blocked mitochondrial fission with the

Drp1 inhibitor peptide P110. P110 specifically inhibits the interac­
tion of Drp1 with Fis1 (23). P110 delivery improved mitochondrial respiration in MCU−/− VSMCs after PDGF treatment and restored mitochondrial fission at baseline and fission in response to PDGF (Fig. 8, A to D). The inhibition of mitochondrial fission also increased mitochondrial Ca2+ uptake in WT and MCU−/− cells (Fig. 8E). In WT VSMCs, overexpression of a constitutively active mutant of CaMKII (CaMKII T287D) induced mitochondrial fission and abrogated fur­
ther fragmentation by PDGF, whereas P110 pretreatment of WT

cells abrogated fission by PDGF (Fig. 8F). When overexpression of

constitutively active CaMKII and Drp1 inhibition with P110 were

combined, increased mitochondrial fusion at baseline and increased

fission after PDGF treatment were observed, substantiating that cytosolic CaMKII is upstream of endogenous Drp1 and enhances mito­

ochondrial fission through Drp1.

Overexpression of mitochondrial dynamin-like GTPase (OPA1) and mitofusin 1 and 2 (MFN1/2), which drive mitochondrial fusion at the inner and outer mitochondrial membranes, increased the mito­

chondrial form factor at baseline in WT and MCU−/− VSMCs. How­
ever, in contrast to the DRP1 inhibitor P110, OPA1 and MFN1/2

overexpression in MCU−/− VSMCs did not recover mitochondrial

fission after PDGF treatment (Fig. 8, G to I), corroborating that the

effects of MCU deletion are through Drp1 activation (Fig. 8J).

DISCUSSION

Here, we demonstrated that MCU deficiency inhibited proliferation

in wound healing, organ development, and postnatal tissue homeo­

stasis in vivo and of smooth muscle and fibroblasts in vitro. In

MCU-deficient cells, cell cycle progression was delayed at the G1−S

transition, a stage in WT cells characterized by mitochondrial fusion and increased mitochondrial Ca2+ uptake. MCU deficiency delayed
cytosolic Ca2+ clearance after ER Ca2+ release, increased activation of CaMKII, Drp1 phosphorylation at Ser616, mitochondrial fission, impaired mitochondrial respiration and glycolysis, and cell prolif­
eration. Mitochondrial OCR and cell proliferation were restored by

either MCU overexpression or inhibition of mitochondrial fission or of cytosolic CaMKII in MCU-deficient cells but not in WT cells.

Thus, we posit that Drp1 phosphorylation occurs physiologically in

response to growth factor treatment of WT cells secondary to tran­
sient activation of CaMKII. When MCU is absent, constitutive acti­
vation of CaMKII promotes constitutive fission, which prevents a

subsequent fusion event that is required for cell cycle progression (Fig. 8J). By controlling cytosolic Ca2+ transients and Ca2+-dependent

signaling mediated by CaMKII, MCU regulates mitochondrial dynamics in G1−S phase and respiration necessary for cell cycle progression.
Although the decreased body weight of MCU−/− mice suggests impaired cell proliferation (12), current data on MCU as a regulator of cell proliferation is limited to cancer cell lines. MCU expression has been positively correlated with poor prognosis, tumor cell migration, invasion, and resistance to apoptosis but not to cell proliferation (24–27). These results may not adequately represent Ca2+ regulation during cell proliferation in nonmalignant cells because oncogene activation alters relevant Ca2+ signaling (28). Changes in the expression or function of Orai3 in breast cancer (29) and of SERCA in prostate cancer (30) imply that cytosolic Ca2+ fluxes are altered at various points during the cell cycle. Nonetheless, inhibition of the LETM1 (leucine zipper/EF hand–containing transmembrane-1 protein), which promotes mitochondrial Ca2+ influx and efflux, impaired fibroblasts proliferation, although the underlying mechanism remains unclear (31).

At G1-S transition, mitochondria are hyperfused and have increased capacity to take up Ca2+ through MCU (6, 11, 13). Here, we demonstrated that MCU was required to maintain cell cycle progression, cytosolic Ca2+ clearance, and mitochondrial hyperfusion during G1-S phase (Figs. 3, A, B, H, and I, and 4, A and B). The mechanisms behind mitochondrial hyperfusion in G1-S transition (13) are not clear. Hyperfusion could be caused by inhibition of Drp1, for example, by dephosphorylation at Ser616 (11) or activation of fusion proteins such as Mfn1/2. Our findings that cytosolic Ca2+ levels increase in WT cells at G1-S (Fig. 4A) imply that mechanisms in addition to Drp1 Ser616 dephosphorylation are in place at this stage. MCU deletion leads to excessive activation of Ca2+/CaMKII/Drp1-dependent fission and thus disrupts these mechanisms. However, we also cannot exclude that other Ca2+-dependent regulators of mitochondrial fission such as calcineurin contribute to the phenotype of MCU−/− cells (16).

Cytosolic CaMKII was activated even in the absence of Ca2+-mobilizing growth factors in MCU-deficient cells. Auto- phosphorylation of CaMKII at Thr287 after an initial Ca2+/CaM burst prevents re-binding of the autoinhibitory segment to the kinase and is the key event by which CaMKII gains Ca2+ independence (32). In MCU-deficient cells, cytosolic Ca2+ clearance was delayed after growth factor application over a prolonged time period (Fig. 2H). Minor differences in baseline cytoplasmic Ca2+ levels were seen as previously reported (10). The delayed cytosolic Ca2+ clearance without increased baseline levels may be sufficient to sustain CaMKII activity and Drp1 Ser616 phosphorylation. CaMKII has been identified as cell cycle promoter, for example, by activating CDC25 in G2 phase (33). CaMKII inhibition in MCU-deficient cells restored PDGF-dependent Drp1 phosphorylation. We posit that this occurs through activation of other kinases that target Drp1 Ser616, such as Erk1/2 (extracellular signal–regulated kinases 1 and 2), CDK1, or the Ca2+-independent PKC isofrom δ (PKCδ) (17). Moreover, mitochondrial OCR increased with CaMKII inhibition even in the absence of MCU, consistent with reports of higher mitochondrial crista number and ATP synthesis activity in cells with hyperfused, tubular mitochondria (34, 35). By contrast, PKCδ inhibition or overexpression of Opa1 and Mfn1/2 did not rescue metabolism or mitochondrial dynamics in MCU-deficient cells.
Fig. 6. Cytosolic CaMKII is activated and controls mitochondrial fission when MCU is deleted. (A) Immunoblots for active CaMKII (CaMKII pThr287) and CaMKII in WT and MCU−/− VSMCs treated with PDGF for 20 min. (B) Quantification of CaMKII phosphorylation at Thr287 normalized to CaMKII protein in PDGF-treated WT and MCU−/− VSMCs as in (A). n = 8 independent experiments. (C) Immunoblots for phosphorylated CaMKII (CaMKII pThr287) and CaMKII in tissue samples from the skin and aorta of WT and MCU−/− mice. (D) Quantification of CaMKII phosphorylation at Thr287 normalized to CaMKII protein in the skin and aorta as in (C). n = 4 independent experiments. (E) Immunoblots for phosphorylated Drp1 at Ser616 (Drp pSer616) and Drp1 protein in WT VSMCs treated with 30 μM KN-93 for 30 min before the addition of PDGF. (F) Quantification of Drp1 phosphorylation at Ser616 normalized to Drp1 protein in (E). n = 3 independent experiments. (G) Confocal microscopy images of mitochondria (mtGFP; green) in WT and MCU−/− VSMCs after adenoviral overexpression of inactive CaMKII (CaMKII T287A) for 48 hours, followed by treatment with PDGF or control for 20 min. Scale bars, 20 μm (larger image) and 5 μm (inset). (H) Quantification of form factor in WT and MCU−/− VSMCs with overexpression of CaMKII T287A or control in (G). n = 5 independent experiments, with the number of cells analyzed indicated in bars. (I) Immunoblots for phosphorylated, active Drp1 at Ser616 (Drp pSer616) and Drp1 protein in WT and MCU−/− VSMCs with overexpression of CaMKII or control for 48 hours, followed by treatment with PDGF or control for 20 min. n = 2 independent experiments. (J) Confocal microscopy images of mitochondria (mtGFP; green) in WT and MCU−/− VSMCs after adenoviral overexpression of CaMKII for 48 hours and treatment with PDGF or control for 20 min. Scale bars, 20 μm (larger image) and 5 μm (inset). (K) Quantification of form factor in WT and MCU−/− VSMCs as in (J). n = 5 independent experiments, with the number of cells analyzed indicated in bars. *P < 0.05, **P < 0.005, ****P < 0.001 by Kruskal-Wallis test (B, H, K, and L), Mann-Whitney U test (D), and two-way repeated measures ANOVA (F).

(Fig. 8H and fig. S7, A and B), supporting that mitochondrial fission and its downstream effects are indeed driven by altered cytosolic Ca2+ levels.

Although numerous cytosolic regulators of mitochondrial dynamics during mitosis have been identified, our data imply that mitochondria through MCU-dependent Ca2+ influx modulate mitochondrial fission and thereby cell proliferation. The MCU complex subunits MICU, EMRE, and MCUB regulate MCU activity (36–38), and the complex composition determines the diverse MCU activity in different tissues (39). In our experiments, we detected evidence for changes in MCU subunit composition that coincided with increased mitochondrial Ca2+ uptake in G1-S phase. In particular, the MCU regulator MCUB (37) and the
subunit EMRE, which is required for opening of the MCU pore and its tight regulation (40, 41), as well as MICU1 (42), were affected. Thus, it is tempting to speculate that acute changes in MCU subunit composition or activity during the cell cycle affect mitochondrial Ca\(^{2+}\) uptake, which positions the MCU complex as an active regulator of cell cycle progression and cell proliferation. Further experiments to ascertain the complex composition in the inner mitochondrial membrane and investigations into the regulation of these events will be necessary to corroborate the role of the MCU complex in this context.

Fig. 7. Cytosolic CaMKII inhibition in MCU\(^{-/-}\) VSMCs rescues mitochondrial dynamics and cell proliferation. (A) OCR in WT and MCU\(^{-/-}\) VSMCs after treatment with PDGF (20 ng/ml for 1 hour) with the sequential addition of oligomycin (Oligo; 1 μM), FCCP (1.5 μM), and rotenone/antimycin (2 μM). (B) ECAR in WT and MCU\(^{-/-}\) VSMCs after treatment with PDGF (20 ng/ml for 1 hour), n = 5 independent experiments for (A) to (C). (C) Quantification of OCR for mitochondrial respiration calculated as baseline OCR – OCR after oligomycin. (D) OCR in WT and MCU\(^{-/-}\) VSMCs with adenoviral overexpression of CaMKII after treatment with PDGF (20 ng/ml for 1 hour). (E) ECAR in WT and MCU\(^{-/-}\) VSMCs with adenoviral overexpression of CaMKII after treatment with PDGF (20 ng/ml for 1 hour). (F) Quantification of OCR for mitochondrial respiration calculated as baseline OCR – OCR after oligomycin. (G) Lactate concentration in WT and MCU\(^{-/-}\) VSMCs after adenoviral overexpression of CaMKII, serum starvation for 24 hours, followed by treatment with growth medium containing PDGF for 24 hours, n = 7 independent experiments. (H) Number of WT and MCU\(^{-/-}\) VSMCs with overexpression of CaMKII and control at 72 hours after PDGF treatment or control. n = 6 independent experiments. Number of biological replicates indicated in bars. *P < 0.05 by Kruskal-Wallis test (C, F, G, and H).
Fig. 8. Inhibition of mitochondrial fission in MCU−/− VSMCs augments mitochondrial Ca2+ uptake, dynamics, and respiration. (A) OCR in WT VSMCs after treatment with P110 (2 μM for 2 hours) and PDGF (20 ng/ml for 1 hour). n = 3 independent experiments. (B) OCR in MCU−/− VSMCs after treatment with P110 and PDGF. n = 3 independent experiments. (C) Confocal microscopy images of mitochondria (mtGFP; green) in WT and MCU−/− VSMCs after treatment with P110 for 2 hours and PDGF or control for 20 min. Scale bars, 20 μm (larger image) and 5 μm (inset). (D) Summary histogram of form factor in control WT and MCU−/− VSMCs. Number of cells analyzed in three independent experiments indicated in bars. (E) Average tracing of mitochondrial Ca2+ by mtPericam in WT or MCU−/− VSMCs after treatment with P110 (2 μM) for 2 hours and acute addition of PDGF (20 ng/ml). n = 3 independent experiments. (F) Summary histogram of form factor in control WT VSMCs with adenoviral overexpression of constitutively active CaMKII [CaMKII T287D; multiplicity of infection (MOI) of 100] or control. Samples pretreated with P110 as indicated. n = 3 experiments, with the number of cells analyzed indicated in bars. (G) Confocal microscopy images of mitochondria (mtGFP; green) in WT and MCU−/− VSMCs after overexpression of OPA1 and MFN1/2 at the baseline and after PDGF treatment. Scale bars, 20 μm (larger image) and 5 μm (inset). (H) Summary histogram of form factor in baseline WT and MCU−/− before and after overexpression of OPA1 and MFN1/2 before and after PDGF treatment. n = 3 independent experiments, with the number of cells analyzed indicated in bars. (I) Immunoblots for OPA-1, Myc (myc-tagged MFN1), MFN2, and GAPDH in WT VSMCs and VSMCs with overexpression of OPA1 and MFN1/2. (J) Graphical summary, indicating that MCU deletion increases CaMKII activation, DRP1 phosphorylation, and mitochondrial fission. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 by Kruskal-Wallis test (D, F, and H).
MATERIALS AND METHODS

Animals

All animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee protocol. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). We used 10- to 16-week-old male and female MCU−/− mice, a gift from T. Finkel, NIH, in CD1 background and littermate controls on regular chow (7913, Teklad) for all in vivo experiments. The body weight in MCU−/− mice and their littermates was determined on postnatal days 7, 18, 21, and 28 and before euthanasia. Weights of the heart, liver, and kidneys were obtained after euthanasia and expressed as ratios of organ to body weight.

Skin wounding

Mice were anesthetized with isoflurane. To create full-thickness skin wounds, mice were shaved, and surgical scrub was performed at the surgical site. A sterile 6-mm biopsy punch was placed on the back, and a punch of dorsol-rostral back skin and panniculus carnosus was excised. Care was taken not to injure underlying muscles. Digital photographs were acquired on the day of surgery and every day thereafter. The wound margins were traced in ImageJ to calculate the wound area (43).

Histology and sectioning

The aortas and livers from 12-week-old MCU−/− and WT littermates were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections of the aorta and liver were collected on SuperFrost Plus slides. H&E staining was performed on sections of the distal thoracic aorta. Deparaffinized liver sections were washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with the Vector Mouse-on-Mouse (MOM) Detection Kit (Vector Laboratories). Anti-PCNA primary antibody was applied for 1 hour at room temperature. Sections were washed with PBS before application of an horseradish peroxidase (HRP)–conjugated secondary antibody for 30 min. 3,3′-Diaminobenzidine was then applied for 10 min. Last, the sections were counterstained with Harris hematoxylin for 10 s.

Cell isolation and culture

Mouse aortic VSMCs were isolated from male and female mice by enzymatic dispersion (44). Cells were cultured in low-glucose (1 mM) Dulbecco’s modified Eagle’s medium (DMEM) with pyruvate supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 8 mM Hepes, MEM vitamins, and nonessential amino acids at 37°C in a humidified 95% air and 5% CO2 incubator. Passage-matched mouse VSMCs from 5 to 10 passages were used for all experiments.

Skin fibroblasts were isolated from 1-week-old male and female MCU−/− and WT littermate mouse pups. (45). After removal of the epidermis, the dermis was cut into small pieces under sterile conditions and digested overnight in DMEM with Fungizone and antibiotics with collagenase type I (300 U/ml). The following day, the digested material was dispersed by vigorous pipetting and centrifuged at 1200 rpm for 5 min. The pelleted cells were plated in DMEM supplemented with 10% FBS, penicillin/streptomycin, and Fungizone (2.5 μg/ml). Cells from passages 1 to 5 were used.

siRNA transfection

Five hundred thousand VSMCs were seeded in a T75 flask overnight. The next day, siRNA duplexes for MCU (5′-CGACCUGAGAAAAUGUAUCCUCGUGAAUCU-UAAUUGTCT-3′ and 5′-GGGAAUAAAGGGAUCU-UAAUUGTCT-3′ at a ratio of 1:1) or scramble control (5′-CGUUAUGCGCU-AUAUACGCGU-3′; Integrated DNA Technologies) were transfected (5 nM in 20 μl of DharmaFECT 4 reagent). Knockdown efficiency was determined after 72 hours by Western blotting for MCU, which was normalized to COXIV signal. The knockdown efficiency was consistently greater than 75%.

Cell counting

VSMCs and skin fibroblasts were cultured in 12-well plates at 5000 cells per well in 2 ml DMEM containing 10% FBS. Twenty-four hours after plating, cells were treated with PDGF (20 ng/ml). RU360 (100 nM) was added with PDGF to some wells. After 24 to 72 hours, cells were trypsinized and counted in triplicate using a Beckman Coulter Z1 cell counter.

TUNEL staining

VSMCs were plated onto eight-chamber microscopy slides. Apoptosis was induced by starvation in serum-free DMEM. After 48 hours, VSMCs were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 10 min. TUNEL staining was performed with a TUNEL staining kit (Roche) and mounted in VECTASHIELD mounting medium with propidium iodide. Ten fields per well were analyzed at 20× using a Zeiss LSM 510 microscope. Data are presented as a percentage of TUNEL-positive nuclei to total number.

Cytosolic Ca2+ imaging

Cells cultured on glass-bottomed tissue culture dishes were washed once with normal Tyrode’s buffer and incubated with 2.5 μM Fura-2 AM (Invitrogen) in loading buffer with 2% bovine serum albumin (BSA) for 20 min at room temperature. Cells were then washed twice with normal Tyrode’s buffer and incubated for another 10 min at 37°C to allow for de-esterification of Fura-2 AM. To record the changes in cytosolic Ca2+ transients, images were acquired continuously, from 3 min before and at least 10 min after PDGF treatment, using an Olympus IX81 Inverted Light microscope. The cells were excited alternatively at 340 and 380 nm. Fluorescence signal intensity was acquired at 510 nm. Data are presented as AUC and peak amplitude. To estimate baseline cytoplasmic Ca2+ levels, two adjacent chambers were created on a glass bottom microwell dish by applying a divider of vacuum grease. Then, VSMCs transfected with MCU siRNA or scrambled control were seeded in the two chambers without mixing the two cell preparations. Fura-2 AM imaging was performed as described above.

Ca2+ retention assay

Calcium Green-5N was used to monitor extramitochondrial Ca2+ in digitonin-permeabilized cultured skin fibroblasts. Recordings of mitochondrial Ca2+ uptake were performed in a 96-well plate with 100 μl of respiration buffer [100 mM K aspartate, 20 mM KCl, 10 mM Hepes, 5 mM glutamate, 5 mM malate, and 5 mM succinate (pH 7.3)] supplemented with 100 μM blebbistatin, 5 μM thapsigargin, 0.005% digitonin, and 1 μM Calcium Green-5N (Invitrogen). Calcium Green-5N fluorescence was monitored at 485-nm excitation and at 535-nm emission after adding CaCl2 (100 μM free Ca2+ at 30°C). FCCP was added at a concentration of 25 μM.
**Adenoviral transduction**

Recombination and amplification of adenoviruses expressing CaMKII (Ad-CaMKII), constitutively active CaMKII (Ad-CaMKII-T287D), mitochondrial-targeted EGFP (enhanced green fluorescent protein; Ad-mtEGFP), or mitochondrial-targeted Pericam (Ad-mtPericam), or empty (Ad-control) were performed by the Gene Transfer Vector Core at the University of Iowa. VSMCs were incubated with adenoviruses at an MOI of 50 in serum-free media overnight. Subsequent experiments were conducted 48 to 72 hours after adenoviral transduction (46).

**Nucleofection**

VSCMs were transfected in a Nucleofector I device (Lonza) with the Basic Nucleofector Kit for Primary Mammalian Smooth Muscle Cells (#VPI-1004, Lonza), following the manufacturer’s protocol. Six hundred thousand cells were transfected in the presence of 5-µg plasmid DNA, plated onto 35-mm glass bottom microwell dishes (MatTek Corporation), and grown for 72 hours before experiments were performed.

**Mitochondrial Ca\(^{2+}\) imaging**

Cells previously transfected with siRNAs for 72 to 96 hours were seeded onto 35-mm glass bottom microwell dishes (MatTek Corporation), infected with mtPericam adenovirus and incubated for 48 hours. Pericam fluorescence was determined in cells in Tyrode’s solution [140 mM NaCl, 10 mM glucose, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 2.0 mM MgCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 5 mM Hepes (pH 7.4) with NaOH]. Imaging was performed at room temperature with a Leica TCS SP8 STED confocal microscope. Pericam was excited at 405 and 480 nm, and its emission was recorded at 535 nm. ATP (100 μM) or PDGF (20 ng/ml) was added by micropipet (in amounts of 10 μl) to trigger mitochondrial Ca\(^{2+}\) uptake. Recordings were performed every 5 s for at least 10 min. mtPericam signals were quantified by ImageJ. The rise in amplitude above baseline and the AUC for 5 min after PDGF application were calculated. Peak amplitude (R) was calculated using \(R_{\text{peak}} - R_{\text{baseline}}\). The AUC was determined by subtracting the AUC at the baseline ratio. Summary data represent the average difference in basal and peak mitochondrial [Ca\(^{2+}\)].

**Cell cycle analysis**

VSMCs transfected with MCU siRNA or scramble or WT and MCU\(^{-/-}\) skin fibroblasts were grown to confluence, maintained in a postconfluent state for 48 hours, and then split to 50% density. DEMEM with 10% FBS was added for the indicated times (24 and 32 hours for VSMCs and 16 and 24 hours for skin fibroblasts). For some experiments, treatment with aphidicolin (2 μg/ml) for 24 hours was used to arrest skin fibroblasts at the G1-S cell cycle transition. After 24 hours, cells were washed with PBS and cultured in DEMEM with 10% FBS and PDGF (20 ng/ml) for 5 hours. The cells were harvested, fixed with 75% ice-cold ethanol, stained with propidium iodide (stock solution of 50 μg/ml), and analyzed in an LSRII Flow Cytometer (Becton Dickinson).

**Cell lysis and fractionation**

For whole-cell lysates, cells were lysed in radioimmunoprecipitation assay buffer [20 mM tris, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (pH 7.4) supplemented with protease (Mini Complete, Roche) and phosphatase inhibitors (PhosSTOP, Roche)]. Lysates were sonicated, and debris were pelleted by centrifugation at 10,000g for 10 min at 4°C. For mitochondrial fraction preparation, cells were washed in PBS and in MSEM buffer (5 mM Mops, 70 mM sucrose, 2 mM EGTA, and 220 mM Mannitol (pH 7.5) with protease inhibitors) before homogenization in cold MSEM buffer using 50 strokes in a Potter-Elvehjem glass Teflon homogenizer. Nuclei and cell debris were pelleted by centrifugation at 600g for 5 min at 4°C. Mitochondria were separated from the cytosolic fraction by centrifugation at 8000g for 10 min at 4°C. Protein concentrations were determined by Pierce BCA protein assay (Thermo Fisher Scientific).

**Immunoblotting**

Equivalent amounts of protein (5 to 15 μg) were separated on NuPAGE 4 to 12% bis-tris gels (Life Technologies) and transferred to polyvinyl difluoride membranes (Bio-Rad). Membranes were blocked in 5% BSA and incubated overnight at 4°C with primary antibodies. Blots were washed three times for 10 min with 0.05% Tween 20 in tris-buffered saline and incubated for 1 hour at room temperature with the secondary antibodies at a dilution of 1:5000 to 1:15,000. Blots were developed with enhanced chemiluminescent (ECL) substrate, scanned, and analyzed using ImageJ software.

**Confocal microscopy**

For immunofluorescence imaging, cells were grown on coverslips precoated with 0.1% gelatin. Mitochondria were labeled by transduction with adenovirus expressing mtGFP for 48 hours or loading with 200 nM MitoTracker Deep Red at 37°C for 30 min, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked in blocking buffer (1× PBS with 2% glycerol, 50 mM ammonium chloride, 5% BSA, and 2% goat serum). Rabbit anti-Drp1 antibody was applied at 1:100 in blocking buffer overnight at 4°C. After rinsing three times in PBS, goat anti-rabbit secondary antibody (1:2000; Alexa Fluor 568) was applied for 1 hour at room temperature. Cells were mounted in VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss LSM 510 confocal microscope equipped with a 63× oil immersion objective, excited with an argon laser at 488 nm filtered with NFT490 and BP505-530 Zeiss filters and with a HeNe laser at 543 nm (NFT565/BP575-615) and controlled by ZEN software (Zeiss). Colocalization of Drp1 with mitochondria was quantified as the Pearson’s coefficient using the JaCoP plug-in for NIH ImageJ software.

**Mitochondrial morphology quantification**

Mitochondrial fission and fusion were determined in VSMCs transduced with adenovirus expressing mtGFP (MOI of 50) for 48 hours. For automated morphometry, images were processed using NIH ImageJ software, with the plug-ins involving either “rolling ball” background subtraction or deblurring by two-dimensional deconvolution with a computed point spread function. Using a custom-written NIH ImageJ macro provided by S. Strack (University of Iowa), processed images were converted to binary (black and white) images by autothresholding, and mitochondrial particles were analyzed for length or form factor [perimeter \(2/(4 \times \pi \times \text{area})\) (47, 48). The parameters for form factor are set with a minimum value of 1 for perfectly circular mitochondria.

**Assessment of bioenergetics**

OCR was monitored with the ESA BioStat Multi Electrode System (ESA Products, Dionex Corporation) in conjunction with a YSI Oxygen Probe (5331) and glass reaction chamber vials in a YSI bath assembly (5301,
Yellow Springs Instruments), all at room temperature. Cells were suspended in Hanks’ balanced salt solution media at a density of 1 × 10^6 to 3 × 10^6 cells per 1 ml; the typical sample size was 2 ml.

For experiments in the Seahorse XF Analyzer, VSMCs were plated onto a 96-well Seahorse plate at a density of 20,000 per well, 24 hours before the experiment. The cells were equilibrated in Seahorse assay medium (unbuffered DMEM) for 1 hours. PDGF (20 ng/ml) was added immediately before the assay in a Seahorse Bioscience XF96 analyzer. Oligomycin, FCCP, and antimycin/rotenone were added to some wells at concentrations of 1, 1.5, and 2 µM, respectively. Total ATP, ADP (adenosine 5’,diphosphate), and AMP (adenosine 5’,monophosphate) were determined with the AMP-Glo Kit (V5011, Promega Corporation) in 10 µg of total cell lysates. All measurements were performed in triplicates. Lactate levels were measured with the Lactate Analyzer (Lactate Scout, EKF Diagnostics) with Lactate Analyzer. Oligomycin, FCCP, and antimycin/rotenone were added to unbuffered DMEM for 1 hours. PDGF (20 ng/ml) was added 16 hours. Cells were trypsinized, counted, and lysed in hypotonic medium (unbuffered DMEM) for 1 hours. PDGF (20 ng/ml) was added 1 hour before the experiment. MitoSOX Red (M36008), DharmaFECT 4 reagent (Thermo Fisher Scientific) were added to VSMCs in a final volume of 1 ml for 30 min and then was added to VSMCs in a final volume of 1 ml for 1 hour before the experiment.

**P110 peptide delivery**
Chariot was used for intracellular delivery of the peptide P110 (synthesized by GenScript) based on the manufacturer’s protocol. The transfection mixture of 100 µl of Opti-MEM, 5 µl of Chariot, and P110 (2 mM) was mixed and incubated at room temperature for 30 min and then was added to VSMCs in a final volume of 1 ml for 1 hour before the experiment.

**Materials**
The following reagents were purchased from Thermo Fisher Scientific: thapsigargin (T7459), recombinant mouse PDGF-BB (PM0044), MitoSOX Red (M36008), DharmaFECT 4 reagent (T-2004-02), and Fura-2 AM (F1221). The TUNEL staining kit (#12156792910) was bought from Roche. Aphidicolin was purchased from Sigma Aldrich. RU360 (#557440, Calbiochem) was applied as a pharmacological inhibitor of MCU.

**Supplementary materials**
Supplementary materials are available with the online version of this article.

**References and Notes**


Loss of MCU prevents mitochondrial fusion in G1-S phase and blocks cell cycle progression and proliferation
Olha M. Koval, Emily K. Nguyen, Velarchana Santhana, Trevor P. Fidler, Sara C. Sebag, Tyler P. Rasmussen, Dylan J. Mittauer, Stefan Strack, Prabhat C. Goswami, E. Dale Abel and Isabella M. Grumbach

DOI: 10.1126/scisignal.aav1439

Balancing Ca2+ pools in proliferating cells
Cell proliferation is an energetically demanding process. During the cell cycle, mitochondrial fusion and mitochondrial Ca2+ uptake increase, both of which correlate with increased ATP production. Koval et al. found that the mitochondrial Ca2+ uniporter (MCU) was required to balance Ca2+ concentrations in the cytosol and mitochondria. Without the MCU, the excess cytosolic Ca2+ resulted in mitochondrial fission mediated by Drp1, reduced ATP output, and decreased cellular proliferation. Thus, the MCU enables ATP production to match energy demands during the cell cycle.

REFERENCES
This article cites 48 articles, 18 of which you can access for free
http://stke.sciencemag.org/content/12/579/eaav1439#BIBL

PERMISSIONS
http://www.sciencemag.org/help/reprints-and-permissions