mPTP opening caused by Cdk5 loss is due to increased mitochondrial Ca\(^{2+}\) uptake

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Abstract

We previously demonstrated that loss of Cdk5 in breast cancer cells promotes ROS-mediated cell death by inducing mitochondrial permeability transition pore (mPTP) opening (Oncogene 37, 1788–1804). However, the molecular mechanism by which Cdk5 loss causes mPTP opening remains to be investigated. Using primary mouse embryonic fibroblasts (MEFs) isolated from Cdk5\(^{-/-}\) mouse embryos, we show that absence of Cdk5 causes a significant increase in both mPTP opening and mitochondrial Ca\(^{2+}\) level. Analysis of subcellular fractions of MEFs demonstrates that Cdk5 localizes in the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) and Cdk5 loss in MAMs causes increased ER-mitochondria tethering, a process required for Ca\(^{2+}\) transfer from the ER to the mitochondria. Loss of Cdk5 also causes increased ATP-mediated mitochondrial Ca\(^{2+}\) uptake from the ER. Inhibition of ER Ca\(^{2+}\) release or mitochondrial Ca\(^{2+}\) uptake in Cdk5\(^{-/-}\) MEFs prevents mPTP opening, indicating that mPTP opening in Cdk5\(^{-/-}\) MEFs is due to increased Ca\(^{2+}\) transfer from the ER to the mitochondria. Altogether, our findings suggest that Cdk5 in MAMs regulates mitochondrial Ca\(^{2+}\) homeostasis that is disturbed upon Cdk5 loss, which leads to mPTP opening.

Introduction

Cyclin dependent kinase 5 (Cdk5) is a small proline-directed serine/threonine kinase that serves various cell functions such as in the regulation of oxidative stress [1, 2], mitochondrial functions [3] and apoptosis [4, 5]. We previously reported that loss of Cdk5 promotes mitochondrial permeability transition pore (mPTP)-mediated apoptosis in breast cancer cells [6]. We found that Cdk5 loss induces mPTP opening, which leads to increased cellular levels of reactive oxygen species (ROS) that subsequently cause increased susceptibility of breast cancer cells to apoptosis. However, the fundamental mechanism by which loss of Cdk5 regulates mPTP opening is unclear.

Mitochondria are cell organelles that execute a number of key cellular functions, including in energy conversion, intermediate cellular metabolism, cell differentiation, immune response, calcium signaling and homeostasis, and cell death. While the outer mitochondrial membrane (OMM) is highly permeable to Ca\(^{2+}\), the inner mitochondrial membrane (IMM) contains the mitochondrial calcium uniporter (MCU) complex that regulates mitochondrial calcium ([Ca\(^{2+}\)]\(_{\text{mit}}\)) influx [7]. As MCU has low affinity to Ca\(^{2+}\) with K\(_d\) of \(\sim 10\mu\text{M}\), high Ca\(^{2+}\) concentration is needed for MCU activity [8]. Mitochondrial uptake of Ca\(^{2+}\) through MCU channels is facilitated by the close proximity of the mitochondria to the endoplasmic reticulum (ER) [9, 10]. A common mechanism by which mitochondria communicate with the ER is through the interface between these two organelles, which is designated as the mitochondria-associated ER membrane (MAM) [11]. MAM regulates transport of lipids, calcium and other metabolites from the ER to the mitochondria [12, 13], and is involved in the regulation of mitochondrial dynamics [14, 15], formation of autophagosomes and cell survival [16, 17]. MAM consists of inositol 1,4,5-trisphosphate receptors (IP3Rs), voltage-dependent anion channels (VDACs), glucose-regulated protein 75 kDa (Grp75), glucose-regulated protein 78 kDa (Grp78) [18], mitofusin 2 (Mfn2), long-chain fatty acid-CoA ligase 4 (FACL-4), phosphofurin acidic cluster sorting protein 2 (PACS-2), B-cell receptor-associated protein 31 (Bap31) and mitochondrial fission 1

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protein (Fis1) [19], and contains a high concentration of calcium [20]. Once Ca\(^{2+}\) is released from the ER to the cytoplasm via the IP3R channels, part of the released Ca\(^{2+}\) is taken up by the mitochondria [21] through the VDACs in the OMM, and the MCU channels in the IMM [10, 22]. Several studies show rapid Ca\(^{2+}\) mobilization from IP3-gated channels to nearby mitochondria, resulting in increased [Ca\(^{2+}\)]\(_{\text{mt}}\) concentration [23–25]. The ER is closely tethered to approximately 5–20% of mitochondrial surface within a 10–30 nm space [26, 27]. This distinct structure is crucial for intracellular calcium homeostasis. Thus, disruption of MAM causes deregulation of calcium mobilization and disruption of [Ca\(^{2+}\)]\(_{\text{mt}}\) homeostasis.

Under physiological conditions, a small rise in mitochondrial Ca\(^{2+}\) concentration leads to increased mitochondrial respiratory chain activity and ATP synthesis [28, 29]. However, pathological mitochondrial calcium overload is associated with increased generation of ROS, mitochondrial depolarization and increased or prolonged mPTP opening [28–30]. The voltage-dependent, high-conductance mPTP channel controls permeabilization of the IMM. While transient opening of mPTP is thought to be a calcium efflux channel in the mitochondria under normal conditions [31, 32], prolonged mPTP opening causes mitochondrial swelling and release of cytochrome C and other intermembrane space (IMS) proteins, leading to activation of caspase-mediated apoptosis [33, 34].

In this study, we used Cdk5\(^{-/-}\) mouse embryonic fibroblasts (MEFs) to investigate how Cdk5 loss induces mPTP opening. We demonstrate that loss of Cdk5 alters ER–mitochondria tethering, increasing mitochondrial Ca\(^{2+}\) uptake from the ER. We propose that Cdk5 loss alters mitochondrial Ca\(^{2+}\) homeostasis, causing mPTP opening.

**Results**

**Cdk5 loss in primary MEFs induces mPTP opening**

Previously, we demonstrated that knocking down Cdk5 by siRNA in breast cancer cells causes mPTP opening and subsequent ROS increase, which promotes cell death [6]. To further characterize the molecular and cellular mechanisms that lead to mPTP opening upon Cdk5 loss, we utilized primary MEFs isolated from wt and Cdk5\(^{-/-}\) mouse embryos as knockout of the Cdk5 gene in mice is associated with perinatal lethality [39]. Initially, we assessed mPTP opening in Cdk5\(^{-/-}\) MEFs by calcine-AM staining followed by treatment with CoCl\(_2\). Calcine-AM is a cell permeable fluorophore that diffuses and gets trapped in all subcellular compartments, including mitochondria [40]. Treatment with cobalt (Co\(^{2+}\)) quenches calcine fluorescence in all subcellular compartments except the mitochondrial matrix which is enclosed by a Co\(^{2+}\) impermeable inner mitochondrial membrane when mPTP is closed. Thus, the ability of Co\(^{2+}\) to quench mitochondrial calcine fluorescence only when mPTP is open allows determination of open vs closed status of mPTP in the cell [40]. As shown in Fig. 1a, fluorescence microscopy of wt and Cdk5\(^{-/-}\) MEFs following calcine staining without CoCl\(_2\) treatment showed strong and similar fluorescence intensity, indicating equivalent intracellular calcine-AM loading. However, upon treatment with CoCl\(_2\), Cdk5\(^{-/-}\) MEFs exhibited less calcine fluorescence intensity compared with wt, indicating greater quenching of mitochondrial calcine fluorescence and thus increased mPTP opening in Cdk5\(^{-/-}\) MEFs compared with wt. Consistent with these observations, flow cytometry analyses of CoCl\(_2\)-treated cells pre-stained with calcine (Fig. 1b, top and bottom panels) showed that Cdk5\(^{-/-}\) MEFs have reduced (p < 0.05) calcine fluorescence intensity compared to wt (0.25 vs 0.52). The clear decline in calcine-stained population of Cdk5\(^{-/-}\) MEFs further indicates greater mPTP opening in these cells compared with wt.

**Loss of Cdk5 in MEFs causes increased level of [Ca\(^{2+}\)]\(_{\text{mt}}\)**

A rise in mitochondrial Ca\(^{2+}\) level, [Ca\(^{2+}\)]\(_{\text{mt}}\), has been shown to be a critical regulator of mPTP opening [41]. Thus, we sought to examine potential alteration in [Ca\(^{2+}\)]\(_{\text{mt}}\) in Cdk5\(^{-/-}\) MEFs by tracing cytoplasmic Ca\(^{2+}\) level, [Ca\(^{2+}\)]\(_{\text{cyt}}\), following the addition of the protonophore and oxidative phosphorylation uncoupler, FCCP. FCCP causes depolarization or collapse of the mitochondrial membrane potential, resulting in mPTP opening and release of Ca\(^{2+}\) from the mitochondria [42]. Therefore, the increase in cytoplasmic Ca\(^{2+}\) level following FCCP treatment in wt as well as Cdk5\(^{-/-}\) MEFs corresponds to [Ca\(^{2+}\)]\(_{\text{mt}}\). To proceed with [Ca\(^{2+}\)]\(_{\text{mt}}\) measurement, wt and Cdk5\(^{-/-}\) MEFs loaded with the cell-permeable intracellular calcium indicator, Fluo-4-AM, were subjected to single cell Ca\(^{2+}\) imaging before and after FCCP treatment. As shown in Fig. 2a, treatment with FCCP caused a greater wave of increase in [Ca\(^{2+}\)]\(_{\text{mt}}\) in Cdk5\(^{-/-}\) MEFs than in wt, indicating increased [Ca\(^{2+}\)]\(_{\text{mt}}\) in Cdk5\(^{-/-}\) MEFs compared with wt. Quantitative analyses revealed a 58% increase (p < 0.05) in peak amplitude (Fig. 2b) and a twofold increase (p < 0.05) in area under the curve (Fig. 2c) in Cdk5\(^{-/-}\) MEFs compared with wt further indicating increased [Ca\(^{2+}\)]\(_{\text{mt}}\) in Cdk5\(^{-/-}\) MEFs.

**Cdk5 is a mitochondria-associated ER membrane (MAM) protein, which when lost induces ER–mitochondria tethering**

The ER is the major intracellular Ca\(^{2+}\) store, and the interface between the ER and the mitochondria, named mitochondria-associated ER membrane (MAM), contains...
channels for calcium transfer [21]. As shown in Fig. 3a, Cdk5 exists in MAMs purified from wt MEFs. Cdk5 as a MAM protein was supported by co-fractionation with the ER-MAM markers, fatty acid-CoA ligase long-chain (FACL4) and glucose-regulated protein 78 kDa (GRP78), and the mitochondria-MAM marker, voltage-dependent anion channel (VDAC). Purity of the isolated MAMs was further assessed by the lack of the nuclear and cytoplasmic markers, proliferating cell nuclear antigen (PCNA) and lactate dehydrogenase (LDH), respectively.

ER-mitochondria tethering is essential for the formation of ER-mitochondria contact sites and subsequent Ca$^{2+}$ transfer from the ER to the mitochondria [43, 44]. To investigate the possibility that ER-mitochondria tethering and contact sites are altered in Cdk5$^{-/-}$ MEFs, causing an increase in [Ca$^{2+}$]$_{mit}$, ultrathin sections of wt and Cdk5$^{-/-}$ MEFs were subjected to TEM (Fig. 3b) as described in the Materials and Methods section. Since the number, length and distance between the ER-mitochondria contact sites have been shown to influence ER Ca$^{2+}$ transfer to the mitochondria [43, 45, 46], we analyzed these parameters in wt and Cdk5$^{-/-}$ MEFs. As shown in Fig. 3c, the percentage of mitochondria that is in contact with the ER is higher (p < 0.05) in Cdk5$^{-/-}$ MEFs than in wt (67% vs 52%). Figure 3d shows that the mitochondrial perimeter that is in contact with the ER in Cdk5$^{-/-}$ MEFs is also greater (p < 0.01) in Cdk5$^{-/-}$ MEFs than in wt (40% vs 15%). In addition, as shown in Fig. 3e, the percentage of ER-mitochondria contact sites with a distance of <30 nm is higher (p < 0.05) in Cdk5$^{-/-}$ MEFs compared with wt (44% vs 25%).

**Loss of Cdk5 causes increased mitochondrial Ca$^{2+}$ uptake from the ER upon stimulation with ATP**

Our next approach was to examine a potential difference in mitochondrial Ca$^{2+}$ uptake from the ER in wt and Cdk5$^{-/-}$ MEFs. To do so, cells in Ca$^{2+}$-free buffer and pre-loaded with a mitochondrial Ca$^{2+}$ probe, Rhod-2 AM [47], were treated with ATP, which binds cell surface purinergic receptors [48] and IP3R [49], which both stimulate...
IP3-evoked ER Ca$^{2+}$ release [49]. As shown in Fig. 4a, treatment with ATP caused a greater wave of increase in [Ca$^{2+}$]$_{mt}$ in Cdk5$^{-/-}$ MEFs than in wt. Quantitative analyses showed a 26% increase ($p<0.05$) in peak amplitude (Fig. 4b), and a 69% increase in integrated Ca$^{2+}$ signal (Fig. 4c) in Cdk5$^{-/-}$ MEFs compared to wt. In addition, the rate of Ca$^{2+}$ influx into the mitochondria was 33% faster in Cdk5$^{-/-}$ MEFs compared to wt (Fig. 4d) while the rate of Ca$^{2+}$ extrusion from the mitochondria was slower by 75% in Cdk5$^{-/-}$ MEFs compared to wt (Fig. 4e). Together, our data indicate that Cdk5$^{-/-}$ MEFs have increased and prolonged ATP-induced mitochondrial Ca$^{2+}$ uptake from the ER compared to wt.

**mPTP opening due to Cdk5 loss is blocked by inhibition of ER Ca$^{2+}$ release or mitochondrial calcium uptake**

We then investigated whether inhibition of either ER Ca$^{2+}$ release with the potent IP3R inhibitor, XeC [50, 51], or mitochondrial calcium uptake with the potent MCU inhibitor, RuR [52], followed by calcein-AM loading and subsequent treatment with CoCl$_2$ would show blockade of increased mPTP opening in Cdk5$^{-/-}$ MEFs. Consistent with our data in Fig. 1, flow cytometry analyses shown in Fig. 5, a, b demonstrate that Cdk5$^{-/-}$ MEFs exhibited less calcein fluorescence intensity compared to wt, indicating greater Co$_{2+}$ quenching of mitochondrial calcein fluorescence and thus increased mPTP opening in Cdk5$^{-/-}$ MEFs compared to wt. However, treatment with XeC or RuR increased the calcein fluorescence intensity in Cdk5$^{-/-}$ MEFs to a level equivalent to that of wt. Treatment with the potent mPTP desensitizers, CsA [53] or SFA [54] also completely prevented mPTP opening in Cdk5$^{-/-}$ MEFs. Cells untreated with CoCl$_2$ and cells treated with ionomycin served as controls. Altogether, we demonstrate that inhibition of either ER Ca$^{2+}$ release or mitochondrial calcium uptake prevents mPTP opening in Cdk5$^{-/-}$ MEFs, further indicating that mPTP opening due to loss of Cdk5 results from increased ER Ca$^{2+}$ transfer to mitochondria.

**Discussion**

We now know that loss of Cdk5 in breast cancer cells causes increased mPTP opening that is associated with mitochondrial depolarization, increased ROS levels, mitochondrial fragmentation and apoptosis [6]. Furthermore, we determined that the mPTP-mediated apoptotic pathway in breast cancer cells is coupled with an increase in both intracellular [Ca$^{2+}$] level and calcineurin activity [6]. In the current study, we identify a novel mechanism by which loss of Cdk5 promotes mPTP opening. Figure 6 illustrates that Cdk5 loss in Cdk5$^{-/-}$ MEFs causes an increase in number and length of ER-mitochondrial contact sites as well as a decrease in gaps between these contact sites. These changes promote an increase in mitochondrial Ca$^{2+}$ uptake from the ER, causing increased [Ca$^{2+}$]$_{mt}$ and subsequent increase in mPTP opening upon loss of Cdk5.

mPTP is a calcium-dependent and CsA-sensitive high-conductance channel. mPTP opening induces permeability of the IMM due to the opening of its pore forming proteins. The components of mPTP and the pore-forming proteins remain unresolved. Nonetheless, several studies implicate physiological roles of mPTP in Ca$^{2+}$ buffering, energy metabolism, and mitochondrial homeostasis. Our group [6] and others [33, 34, 55, 56] also showed that increased or prolonged mPTP opening results in mitochondrial depolarization,
increased ROS generation, inhibition of ATP synthesis, and release of apoptosis-inducing mitochondrial proteins such as cytochrome C and apoptosis inducing factor, causing activation of the caspase-mediated apoptotic pathway. We observed these mPTP opening-induced characteristics in breast cancer cells lacking Cdk5 but Cdk5/K−/− MEFs also exhibit increased mPTP opening, which is reversed by mPTP desensitizers such as CsA and SFA and show increased [Ca²⁺]mit level that has been shown to induce mPTP opening. As with breast cancer cells depleted of Cdk5 by siRNA, we determined here that

Fig. 3 Cdk5 localizes in MAMs and loss of Cdk5 causes increased number and length of ER-mitochondria contact sites and reduced contact distance between these two organelles. a Immunoblot analysis of purified MAM and mitochondria in wt and Cdk5/K−/− MEFs. 50μl of homogenate (H), crude MAM (C-MAM), purified MAM (P-MAM), and purified mitochondria (MT) were loaded. For subcellular markers, fatty acid-CoA ligase, long-chain (FACL4) and glucose-regulated protein 78 kDa (GRP78) were used for ER-MAM; VDAC for mitochondria-MAM; proliferating cell nuclear antigen (PCNA) for nucleus; and lactate dehydrogenase (LDH) for cytoplasm. Blots shown represent data from one of three independent experiments (n=3) showing similar results. b–e Transmission electron microscopy (TEM) of ER-mitochondria tethering in wt and Cdk5/K−/− MEFs. Images were acquired using a Hitachi H7650 TEM. Scale bar = 100 nm. ER: endoplasmic reticulum; M: mitochondria. Arrowheads are directed at ER-mitochondria contact sites. For the morphometric analysis in c–e, a total of six TEM grids (3.05 mm discs) prepared from two embeddings per genotype were analyzed: four grids from embedding 1 and two grids from embedding 2. Mitochondrial counts and mitochondria-ER contact measurements were performed in groups of two grids (n = 3, where replicates 1 and 2 originate from the four grids from embedding 1 and replicate 3 originates from the two grids from embedding 2. In c, all observed mitochondria in wt and Cdk5/K−/− MEFs, irrespective of the number of cells, were counted and the percentage of mitochondria in contact with ER was calculated. In d, the mitochondrial perimeter in contact with ER was measured and the percentage of the total individual mitochondrial perimeter in contact with ER was calculated. In e, distances between ER and mitochondria in contact sites were measured and the percentage of the total contact sites with different ranges of ER-mitochondria distances were calculated. In d and e, a total of at least 40 ER-mitochondria contacts, irrespective of the number of cells, were analyzed per two TEM grids (i.e., at least 120 ER-mitochondria contacts were analyzed per genotype). Embeddings were from different cultures of MEFs. Values are means ± SEM; n = 3; *p < 0.05 and **p < 0.01 by unpaired Student’s t test.
Cdk5−/− MEFs exhibit increased mPTP opening that is reversed by the mPTP desensitizers, CsA and SFA, and show increased \([\text{Ca}^{2+}]_{\text{mt}}\) level that induces mPTP opening. It is interesting, however, that while loss of Cdk5 in breast cancer cells promotes apoptosis, we observed that Cdk5−/− MEFs show increased proliferation (data not shown). This was unexpected but supports differential physiology and roles of Cdk5 in normal and cancer cells, and calls for more comprehensive investigations on the function of Cdk5 in mitochondrial calcium dynamics in normal and disease conditions.

MAM, a proteinaceous link between the ER and mitochondria, is important for calcium and metabolite transfer between these organelles. In this study, we show that Cdk5 is enriched in pure MAM fractions, a finding that is consistent with the mass spectrometry analysis by Poston et al., identifying, with high confidence, Cdk5 as one of the MAM proteins in mouse brain [57]. For effective calcium transfer, MCU channels require close ER-mitochondria apposition that is influenced by the ER-mitochondria contact site number, length and distance. The ER-mitochondria contact sites allow the rapid transfer of large amounts of \([\text{Ca}^{2+}]_{\text{mt}}\) from the ER to the mitochondria upon opening of the IP3-gated channels [23, 24, 58]. The distance from the OMM to the rough and smooth ER was determined to be 10–30 nm and 9–16 nm respectively [45, 59], and about 5–20% of the mitochondrial surface is closely apposed to the ER [26, 27]. Our studies show that in wt cells, the ER is juxtaposed to 15% of the mitochondrial surface whereas loss of Cdk5 increases the ER-mitochondrial juxtaposition to about 40%. Similarly, the percentage of ER-mitochondria contacts within ≤30 nm is higher in the absence of Cdk5. Since disruption in ER-mitochondria tethering alters \([\text{Ca}^{2+}]_{\text{mt}}\) transfer between the ER and the mitochondria [60], it is not surprising that we observed increased ATP-induced mitochondrial calcium uptake in cells lacking Cdk5. Similarly, prolonged extrusion of mitochondrial \([\text{Ca}^{2+}]_{\text{mt}}\) in the absence of Cdk5 is expected to contribute to \([\text{Ca}^{2+}]_{\text{mt}}\) accumulation and subsequent mPTP opening. Nevertheless, our observations indicate that Cdk5 in MAM serves to control ER-mitochondrial tethering, and loss of Cdk5 causes an upsurge in mitochondrial calcium uptake by increasing ER-mitochondrial tethering. While studies are underway to

**Fig. 4** Stimulation with ATP causes greater mitochondrial \([\text{Ca}^{2+}]_{\text{mt}}\) uptake from the ER in Cdk5−/− MEFs compared to wt. **a** Wt and Cdk5−/− MEFs loaded with Rhod-2 AM (5 µM) for 30 min in \([\text{Ca}^{2+}]_{\text{free}}\) buffer were analysed by single cell \([\text{Ca}^{2+}]_{\text{mt}}\) imaging. ATP (1 µM) was added where indicated. Data show mean values of \([\text{Ca}^{2+}]_{\text{mt}}\) signals from 15 randomly selected cells and represent one of three independent experiments showing a similar results. Analysis of results from the three independent experiments (\(n = 3\)), each with data from 10–15 randomly selected cells from each genotype, showed that compared to wt, Cdk5−/− MEFs exhibited: **b** higher peak amplitude (\(F/F_0\)) corresponding to greater \([\text{Ca}^{2+}]_{\text{mt}}\) influx into the mitochondria; **c** increased integrated \([\text{Ca}^{2+}]_{\text{mt}}\) signals (area under the curve from 120 to 600 s), which also indicates elevated \([\text{Ca}^{2+}]_{\text{mt}}\) entry into the mitochondria; **d** reduced time of \([\text{Ca}^{2+}]_{\text{mt}}\) influx into mitochondria, indicating faster rate of influx; and **e** increased time of efflux, indicating slower rate of efflux. Values are means ± SEM. ∗∗∗∗< 0.05 by unpaired Student’s t test.
further characterize the specific role of MAM Cdk5 in ER-mitochondria tethering, our finding that inhibition of either ER Ca^{2+} release with XeC or mitochondrial calcium uptake with RuR blocks the increase in mPTP opening induced by Cdk5 loss suggests that elevated mitochondrial calcium influx underlies the increase in mPTP opening in the absence of Cdk5. Altogether, our studies demonstrate a novel mechanism whereby Cdk5 controls mPTP opening by regulating the formation of ER-mitochondria contact site structures and subsequently, calcium transfer from the ER to the mitochondria and [Ca^{2+}]_{mt} level.

Materials and methods

Materials

Dulbecco’s modified eagle’s medium (DMEM), heat-inactivated fetal bovine serum (FBS), EDTA-Trypsin, antibiotic-antimycotic, Calcein AM, Fluo-4 AM and Rhod-2 AM were from Life Technologies Inc. The protease inhibitor cocktail, Cyclosporine A (CsA), Ruthenium Red (RuR), ATP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and Xestospongin C (XeC) were from Sigma. Antibodies to Cdk5 (C-8), FAACL-4 (F-4), VDAC1 (B-6), GRP78 (A-10), PCNA (PC-10) and LDH-A (E-9) were from Santa Cruz Biotech. Sanglifehrin A (SFA) was a gift from Novartis (Switzerland).

Primary MEF isolation and culture

Primary MEFs were isolated from E13.5 wt and Cdk5^{−/−} mouse embryos following the protocol described by Durkin et al. [35] which was based on the method by Todaro and Green [36] and subsequent modifications of the technique by Coats et al. [37]. Embryos were from Cdk5^{+/−} pregnant female mice crossed with Cdk5^{−/−} male mice. Embryos were washed with (phosphate-buffered saline) PBS, decapitated and eviscerated then washed again with PBS. Embryos were minced with sterile forceps and placed in 3–5 ml of 0.05% trypsin-EDTA, pipetted up and down to get cells into suspension and incubated at 37 °C for 5 min. Cell suspensions were transferred to new tubes containing MEF medium (DMEM-high glucose supplemented with 10% FBS, 1% penicillin-streptomycin and 2 mM GlutaMAX) then centrifuged at 1,000 rpm for 5 min. Cell pellets were resuspended in fresh media and plated in 10 cm cell culture dishes. Primary MEFs were cultured in DMEM supplemented with 10% FBS and 100 U/ml each of penicillin and streptomycin under hypoxic condition (5% O_{2} and 5% CO_{2} incubator). All experiments were performed in passage P2-P7 MEFs.

Transmission electron microscopy

TEM analysis was performed following fixation, dehydration, infiltration, and embedding of wt and Cdk5^{−/−} MEFs in situ. Ultra-thin (~60 nm) sections were cut and stained with 2% uranyl acetate and lead citrate and observed under a Hitachi H7650 TEM at the University of Calgary’s Microscopy and Imaging Facility. Images were acquired through an AMT 16000 CCD mounted on the microscope.
MAM and mitochondria isolation

Crude and pure MAMs, and mitochondria were isolated using Percoll density gradient centrifugation as described previously [38]. Briefly, trypsinized MEFs were homogenized in 10 ml of IBcells-1 buffer containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, and 30 mM Tris–HCl (pH 7.4). Homogenates (H) were centrifuged at 600 × g for 5 min at 4 °C. The resulting supernatants were subjected to further centrifugation at 7000 × g for 10 min at 4 °C. Pellets were resuspended in 10 ml of IBcells-2 buffer containing 225 mM mannitol, 75 mM sucrose and 30 mM Tris–HCl (pH 7.4) followed by centrifugation at 10,000 × g for 10 min at 4 °C. The resulting pellets were resuspended in 2 ml of mitochondria resuspending buffer (MRB: 250 mM mannitol, 0.5 mM EGTA and 5 mM HEPES, pH 7.4). The crude mitochondria were then layered with 8 ml of Percoll medium [225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA and 30% Percoll] and 4 ml of MRB then centrifuged at 95,000 × g for 15 min at 4 °C and incubated with 1 µM calcein-AM, 200 nM MitoTracker Red, treated with or without CoCl2 (1 mM) in Hank’s balanced salt solution (HBSS), and incubated for 15 min at 37 °C under 5% CO2. Cells were then washed with HBSS buffer and images were taken under an Olympus 1 × 71 fluorescence microscope using a x10 objective with or without the ×1.6 magnification changer.

Western blotting

Isolated subcellular fractions (50 µl) were loaded in 12.5% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% skimmed milk and then incubated with the indicated primary antibody (1:1000 dilution) at 4 °C overnight. After washing with Tris-buffered saline (TBS) + Tween-20 (TBST) (50 mM Tris–HCl, pH 7.6, 0.1% Tween-20, 0.8% NaCl), membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 h. Immunoreactive bands were detected using ECL reagent (Amersham).

mPTP opening assay

Calcine fluorescence was measured using the Image-IT live mitochondria permeability transition pore assay kit (Thermo Fisher Sci.) as per the manufacturer’s protocol. Briefly, cells seeded on a four-chamber cover glass (Lab-Tek) were loaded with calcine AM (1 µM), MitoTracker Red (200 nM), treated with or without CoCl2 (1 mM) in Hank’s balanced salt solution (HBSS), and incubated for 15 min at 37 °C under 5% CO2. Cells were then washed with HBSS buffer and images were taken under an Olympus 1 × 71 fluorescence microscope using a x10 objective with or without the ×1.6 magnification changer.

For flow cytometry, cells (1 × 10^5) were harvested using Trypsin-EDTA and pre-incubated with CsA (1 µM), SFA (2 µM), XeC (3 µM) or RuR (3 µM) for 1 h then stained as indicated above. Cells were then washed with HBSS, resuspended in ice-cold PBS and analyzed by flow cytometry using a fluorescein isothiocyanate filter (530 nm) for measuring calcine fluorescence.

Calcium measurement

For cytoplasmic Ca^{2+} measurements, MEFs seeded on 3.5 cm glass bottom petri dishes (80% confluence) were loaded with Fluo-4 AM (5 µM) in HBSS (with 1.26 mM calcium) for 30 min at RT. Cells were washed three times with KRH buffer (125 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 25 mM HEPES, 6 mM glucose) then subjected to single cell calcium
imaging using a Leica TCS SP8 confocal microscope (×20 objective). Fluorescence signals were quantified as ratio ($F/F_0$) of the fluorescence after addition of FCCP, relative to the basal fluorescence ($F_0$) before stimulation.

For mitochondrial Ca$^{2+}$ measurements, MEF cells seeded on 3.5 cm glass bottom petri dishes (80% confluency) were loaded with Rhod-2 AM (5 μM) in KRH buffer (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose) for 30 min at RT. Cells washed three times with KRH buffer were subjected to single cell calcium imaging using a Zeiss LSM 510 Meta laser scanning confocal microscope (×20 objective). Fluorescence signals were quantified as ratio ($F/F_0$) of the fluorescence after addition of ATP, relative to the basal fluorescence ($F_0$) before stimulation.

**Statistical analysis**

Student’s $t$ test (unpaired, two-sided) was used. Significance was set at $p < 0.05$.

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**Compliance with ethical standards**

Conflict of interest The authors declare that they have no conflict of interest.

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