Cdk5 regulates IP3R1-mediated Ca\(^{2+}\) dynamics and Ca\(^{2+}\)-mediated cell proliferation

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Abstract
Loss of cyclin-dependent kinase 5 (Cdk5) in the mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs) increases ER–mitochondria tethering and ER Ca\(^{2+}\) transfer to the mitochondria, subsequently increasing mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{mt}}\)). This suggests a role for Cdk5 in regulating intracellular Ca\(^{2+}\) dynamics, but how Cdk5 is involved in this process remains to be explored. Using ex vivo primary mouse embryonic fibroblasts (MEFs) isolated from Cdk5\(^{-/-}\) mouse embryos, we show here that loss of Cdk5 causes an increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)), which is not due to reduced internal Ca\(^{2+}\) store capacity or increased Ca\(^{2+}\) influx from the extracellular milieu. Instead, by stimulation with ATP that mediates release of Ca\(^{2+}\) from internal stores, we determined that the rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in Cdk5\(^{-/-}\) MEFs is due to increased inositol 1,4,5-trisphosphate receptor (IP3R)-mediated Ca\(^{2+}\) release from internal stores. Cdk5 interacts with the IP3R1 Ca\(^{2+}\) channel and phosphorylates it at Ser421. Such phosphorylation controls IP3R1-mediated Ca\(^{2+}\) release as loss of Cdk5, and thus, loss of IP3R1 Ser421 phosphorylation triggers an increase in IP3R1-mediated Ca\(^{2+}\) release in Cdk5\(^{-/-}\) MEFs, resulting in elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\). Elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\) in these cells further induces the production of reactive oxygen species (ROS), which upregulates the levels of Nrf2 and its targets, Prx1 and Prx2.

Cdk5\(^{-/-}\) MEFs, which have elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\), proliferate at a faster rate compared to wt, and Cdk5\(^{-/-}\) embryos have increased body weight and size compared to their wt littermates. Taken together, we show that altered IP3R1-mediated Ca\(^{2+}\) dynamics due to Cdk5 loss correspond to accelerated cell proliferation that correlates with increased body weight and size in Cdk5\(^{-/-}\) embryos.

Keywords Proliferation · Cdk5 · Ca\(^{2+}\) signaling · IP3R

Introduction
Cdk5 belongs to the Cdk family of small serine/threonine kinases, which, together with their respective cyclin activators, regulate the eukaryotic cell cycle [1]. It was identified based on its structural similarity to Cdk1 (Cdc2) and Cdk2 [2–4], but most Cdk5 studies are directed at non-cell cycle events. Nonetheless, there is increasing evidence implicating a role for Cdk5 in cell cycle progression and proliferation [5–8]. For example, in human HeLa cervical epithelial cells, Cdk5 and its activator, p35, have been mapped to centrosomes and suggested to regulate centrosome-mediated cell cycle events [9]. In addition, Cdk5 was found to suppress the neuronal cell cycle [6, 10, 11], particularly at G\(_{1}\)/S [5, 8, 10], and in non-neuronal cells, Cdk5 was found to be required for intra-S and G\(_{2}\)/M cell-cycle checkpoints [7]. Cdk5 regulates cell-cycle progression by downregulating p21\(^{CIP1}\) [1, 12, 13] and p27\(^{KIP1}\) [1, 13], and cell proliferation through modulation of AKT [13, 14], STAT3 [15, 16] or ERK5 [17]. However, gaps remain in our understanding of how Cdk5 regulates the cell cycle and cell proliferation.

The manner in which the various intracellular Ca\(^{2+}\) channels, pumps and exchangers are distributed allows extracellular stimuli to induce [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations in a highly defined spatial and temporal patterns, inducing specific cellular responses such as cell proliferation [18, 19]. Increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) are triggered through a number of mechanisms, including entry from the extracellular milieu, reduced internal Ca\(^{2+}\) store capacity, and Ca\(^{2+}\) release from internal stores, primarily the endoplasmic reticulum (ER). The role of Cdk5 in regulating intracellular Ca\(^{2+}\) dynamics has...
been primarily examined in neurons where it is abundantly expressed [3, 20]. For example, neuronal Cdk5 has been implicated in regulating external Ca^{2+} entry from the extracellular milieu. Cdk5 phosphorylates the P/Q-type voltage-dependent Ca^{2+} channel (VDCC), supressing external Ca^{2+} entry [21]. Cdk5 also phosphorylates the N-type VDCC [22] and the transient receptor potential cation channel subfamily V member 1 (TrpV1) [23–26], stimulating Ca^{2+} influx from the extracellular milieu. In nonneuronal cells, Cdk5-mediated phosphorylation of the purinergic P2X receptor 2 (P2X2)'s full-size isoform (P2X2aR) at Thr372 stimulates mediated phosphorylation of the purinergic P2X receptor 2 from the extracellular milieu. In nociceptive neurons, Cdk5 phosphorylation of the P2X2aR also decreases Ca^{2+} fluxes and subsequently neuronal cell death. Cdk5-mediated tau phosphorylation also causes an increase in Ca^{2+} transfer to the mitochondria, subsequently increasing [Ca^{2+}]_{mt}. This points to a role for Cdk5 in regulating intracellular Ca^{2+} dynamics using the Cdk5 inhibitors, roscovitine (ros, IC_{50} = 0.65 µM; olo, IC_{50} = 25 µM) and olomoucine (olo, 100 µM) [31]. However, the concentrations used to inhibit Cdk5 in this study lack specificity as other kinases such as Cdk1 (ros, IC_{50} = 0.65 µM; olo, IC_{50} = 7 µM), Cdk2 (ros, IC_{50} = 0.7 µM; olo, IC_{50} = 7 µM) and ERK1 (ros, IC_{50} = 34 µM; olo, IC_{50} = 25 µM) could have also been inhibited. In non-neuronal cells, the specific role of Cdk5 in regulating intracellular Ca^{2+} dynamics remains unknown.

The IP3R, an ER transmembrane protein that forms a Ca^{2+} channel in its transmembrane domain and an IP3-binding site on its cytosolic face [32], forms the major route for Ca^{2+} release from the ER. When extracellular soluble agonists bind a G protein-coupled receptor, phospholipase C (PLC) is activated, producing IP3 from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 binding to IP3R induces opening of this channel and release of Ca^{2+} from the ER. Ca^{2+} released from the ER is then mobilized to the mitochondria [33] through the voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane (OMM) and the mitochondrial calcium uniporter (MCU) channels in the inner mitochondrial membrane (IMM). In previous studies [34], we demonstrated that loss of Cdk5 in MEFs increases ER–mitochondria tethering and ER Ca^{2+} transfer to the mitochondria, subsequently increasing [Ca^{2+}]_{mt}. This points to a role for Cdk5 in regulating intracellular Ca^{2+} dynamics. Indeed, Cdk5 localizes in the MAM ER-mitochondria interface [34], and thus is well placed to influence ER Ca^{2+} release through IP3R, which is regulated by IP3R phosphorylation [18]. Cdk5 has a preferred phosphorylation consensus sequence of (S/T)(P)(K/H/R) [3], and among the IP3R isoforms, IP3R1 has two potential Cdk5 phosphorylation sites: S_{421}PLK and T_{799}PVK [35]. IP3R2 is insensitive to ATP and does not contain possible Cdk5 phosphorylation sites; IP3R3 has Thr_{799}, but ATP-induced, IP3R3-mediated Ca^{2+} release is much less significant than that mediated by IP3R1 [36]. It is possible that Cdk5 interacts with and phosphorylates IP3R1, regulating its channel opening. In fact, the Cdk5-related kinase, Cdk1 phosphorylates IP3R1 at Thr_{799} causing IP3R1 opening [35, 37]. Conversely, ERK1/2 phosphorylation of IP3R1 at Ser_{436} decreases IP3 binding and thus IP3-induced Ca^{2+} release [38–40].

Interplay between [Ca^{2+}]_{cyt} and ROS signaling has been reported [41, 42]. Cellular ROS are metabolic byproducts and act as secondary messengers in signaling pathways at sub-toxic levels. Oxidative stress, however, activates the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor by inhibiting its negative regulator, Keap1. Nuclear translocation of activated Nrf2 results in the production of the antioxidant enzymes, peroxiredoxins (Prx1 and Prx2), catalase, glutathione peroxidase (GPX), and heme oxygenase-1 (HO-1), to maintain optimal cellular redox balance [43].

In this study, we utilized the Cdk5^{−/−} mouse model and corresponding ex vivo MEFs to explore the mechanisms by which Cdk5 regulates intracellular Ca^{2+} dynamics and Ca^{2+}-mediated cell proliferation. We demonstrate that Cdk5 targets IP3R1 to control ER Ca^{2+} release and [Ca^{2+}]_{cyt}. These Cdk5-mediated Ca^{2+} dynamics are reflected in the disrupted Ca^{2+}-mediated proliferation of Cdk5^{−/−} MEFs and development of Cdk5^{−/−} embryos.

**Materials and methods**

**Materials**

Dulbecco’s modified eagle’s medium (DMEM), heat-inactivated fetal bovine serum (FBS), EDTA-Trypsin, antibiotic–antimycotic, H_{2}-DCFDA, MitoSOX red, MitoTracker green, Fluo-4 AM, Mag-Fluo-4 AM, ionomycin, GlutaMAX and an antibody against p27KIP1 (719,600) were from ThermoFisher Scientific (Waltham, MA). Mito-tempo, Prx1 (N-19) and actin (I-19) were from Santa Cruz Biotech (Manassas, VA, USA). The polyclonal antibodies against the two IP3R1 phosphopeptides, MLKIGTspS_{421}VKEDKE and HVDRDPQEQVpT_{799}PVK, were generated by GL Biochem. Ltd (Shanghai, China). The
phosphoThr202/Tyr204-ERK1/2 antibody was from Cell Signaling (Danvers, MA, USA). The Ki67 (ab92353), Prx2 (ab109367), GAPDH (6C5) and Nrf2 (ab31163) antibodies and BAPTA-AM (ab120503) were from Abcam (Cambridge, MA, USA). The protease inhibitor cocktail, ATP and XeC were from Sigma (ON, Canada). Thapsigargin (TG) was a gift from Dr. Andrew Braun at the University of Calgary. The IP3R1 siRNAs were synthesized at the University of Calgary Core DNA Services. The peroxidase and serum-free protein block kits were from Dako (Glostrup, Denmark). The avidin and biotin block kit and DAB were from Zymed (CA, USA). The secondary antibodies were from Jackson Immunoresearch Labs (Pennsylvania, USA). The Vectastain® ABC Reagent was from Vector Laboratories (CA, USA). ECL reagent was from GE Healthcare (Chicago, USA). Jet prime transfection reagent was from Polyplus transfection (NY, USA).

Animals

The Cdk5−/− embryos that we used in our studies were generated by intercrossing Cdk5−/− mice (Jackson Laboratory, Bar Harbor, ME, USA) maintained at the University of Calgary Animal Facility. Wt littermates were used as controls. All animal studies conformed to regulatory standards and were approved by the University of Calgary Health Sciences Animal Care Committee.

Isolation and culture of primary MEFs

Primary MEFs were isolated from E13.5 Cdk5+/+ and Cdk5−/− embryos as described previously [34]. Briefly, embryos were washed with 1× PBS, decapitated and eviscerated, and then washed again with PBS. Embryos were minced using 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 tubes containing MEF medium (DMEM-high glucose supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin, and 2 mM GlutaMAX) and then centrifuged at 1000 rpm for 5 min. Cell pellets were resuspended in fresh media and plated in 10 cm cell culture dishes. Primary MEFs were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin under hypoxic condition (5% O2 and 5% CO2) at 37 °C. All experiments were performed using passage 2 to 7 (P2-P7) MEFs.

Ca2+ measurement

(i) To measure resting [Ca2+]cyt, trypsinized 0.5×10⁶ MEFs were loaded with 5 μM Fluo-4 AM in DMEM for 1 h at room temperature. Cells were then washed three times with Ca2+-free EGTA-containing KRH buffer (25 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1.2 mM MgCl2 and 6 mM glucose) and analyzed using a Shimadzu RF 5301PC spectrofluorometer. To minimize background fluorescence, 40 μM EDTA was added before reading the F values. Fmax value was obtained after treatment with 0.02% saponin and addition of 2 μM CaCl2 three times (Supplementary Fig. 1A). Fmin value was taken upon addition of 4 mM EDTA. [Ca2+]cyt was calculated using the formula: free [Ca2+]cyt = Kd [F−Fmin]/[Fmax−F] [44], whereas Kd (for Fluo-4) = 345 nM. (ii) To measure [Ca2+]cyt transients by single-cell Ca2+ imaging, MEFs were seeded in 3.5 cm glass bottom petri dishes and stained with 5 μM Fluo-4 AM in HBSS (with 1.26 mM Ca2+) for 30 min at room temperature (RT). Cells were then washed three times with KRH buffer and analyzed by single-cell Ca2+ imaging using a Zeiss LSM 510 Meta confocal laser scanning microscope (20× objective). Fluorescence signals were measured in 10–20 cells. Peak amplitudes were quantified as ratios of fluorescence (F/F0) after addition of ATP, XeC or TG. F0 represents basal fluorescence or fluorescence before stimulation. (iii) To measure ER Ca2+, MEFs were seeded in 3.5 cm glass bottom petri dishes and stained with 5 μM Mag-Fluo-4 AM in DMEM for 30 min at RT. Cells were then permeabilized with 0.1 mg/ml saponin for 45 s, washed with ICM buffer (10 mM HEPES, pH 7.4, containing 19 mM NaCl, 125 mM KCl, 1.5 mM Na2ATP, 0.735 mM MgCl2, 1 mM EGTA, 0.5 mM CaCl2) three times and analyzed using a Zeiss LSM 510 Meta confocal microscope (20× objective). Fluorescence signals were measured in ten cells and quantified as ratios of fluorescence (F/F0) after addition of 500 nM IP3. F0 represents basal fluorescence or fluorescence before stimulation.

siRNA transfection

MEFs (2.5×10⁶) seeded in 6 cm dishes were transfected with 100 nM IP3R1 siRNA #1 (AACATTGTGCAGAAA ACAGCC) or #2 (AACAAGAGATCCGTAGTAA) for 48 h using Jet prime transfection reagent following the manufacturer’s protocol.

Transfection of S421A and S421D IP3R1

pcDNA 3.0 carrying rat IP3R1 was obtained from Dr. I. Bezprozvanny at the University of Texas Southwestern Medical Center at Dallas. S421A IP3R1 and S421D IP3R1 were custom-generated by Genscript (USA). The IP3R also carries silent mutations: 2118 G > A, 2121 C > A, 2122 C > A, 2124 T > G, 2125 A > T and 2126 G > C that do not alter the IP3R1 amino acid sequence but confer resistance to IP3R1 siRNA #2. MEFs (5×10⁵) seeded in 6 cm dishes were co-transfected with IP3R1 siRNA and
(100 nM) and pcDNA 3.0 carrying S421A IP3R1 or S421D IP3R1 (2 µg) as per the Lonza nucleofector protocol (Basel Switzerland).

**ROS measurement**

(i) For live-cell imaging, MEFs seeded in 4-chamber cover glass (Lab-Tek) were stained with 5 µM DCFDA or 200 nM MitoTracker green + 5 µM MitoSOX red to measure cytoplasmic hydrogen peroxide or mitochondrial superoxide levels, respectively. Images were taken using an Olympus 1X71 fluorescence microscope at 160× magnification. (ii) By flow cytometry, MEFs (2.5 × 10⁵) seeded in 3.5 cm dishes were treated with 3 µM XeC, 10 µM ionomycin or 50 µM BAPTA-AM for 30 min. Cells were then washed with KRH buffer and harvested using trypsin. Cytoplasmic hydrogen peroxide and mitochondrial superoxide levels were measured by staining with 5 µM H₂DCFDA and 5 µM MitoSOX red, respectively, in KRH buffer for 30 min at 37 °C. Cells were then washed and resuspended in KRH buffer and analyzed by flow cytometry using a fluorescein isothiocyanate filter (530 nm) for DCFDA, a phycoerythrin filter (575 nm) for MitoSOX red.

**Proliferation analysis**

1 × 10³ MEFs were seeded in 96 well plates (n = 3). Cdk5−/− MEFs were treated (or untreated) with 3 µM XeC. After 1, 3 or 6 days in culture, cells were harvested using trypsin and stained with trypan blue, and viable cells were counted using a hemocytometer under an Olympus CK40 microscope.

**Immunoprecipitation and immunoblotting**

Immunoprecipitation (IP) of clarified MEF lysates in lysis buffer (25 mM HEPES, pH 7.4, containing 250 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml antipain and 15 µg/ml benzamidine) was performed using IP3R1 (E-8) antibody. IP samples or cell lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and proteins were transferred onto nitrocellulose membranes, which were blocked in 5% skimmed milk and then incubated with the indicated primary antibody at 4 °C overnight. After washing with TBST buffer, containing 50 mM Tris–HCl, pH 7.6, 0.8% NaCl and 0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were detected using ECL reagent (Amersham).

**Immunohistochemistry**

E13.5 mouse embryos fixed in 4% paraformaldehyde (PFA) were sectioned to 10 µm thickness using a Leica RM2235 microtome. Tissue sections mounted on glass slides were incubated initially with peroxidase and then with avidin and biotin using a block kit followed by a serum-free protein block kit to eliminate non-specific binding of the primary antibody. The slides were then incubated with a Ki67 antibody at 1:100 dilution for 2 h, washed, and incubated with a secondary antibody for 40 min. Ki67-positive cells were detected by incubating with Vectastain® ABC reagent for 30 min followed by DAB for 5 min. Tissue staining was visualized and photographed using an Olympus IX71 light microscope with an attached Photometrics Coolsnap FX camera from Roper Scientific (Arizona, USA).

**Statistical analysis**

Cdk5-regulated (i) [Ca²⁺]cyt effect on ROS level, (ii) proliferation, and (iii) IP3-induced Ca²⁺ release were analyzed by one-way ANOVA. All other data were analyzed by Student’s t test. Significance was set at p < 0.05.

**Results**

**Elevated ATP-evoked rise in [Ca²⁺]cyt in Cdk5−/− MEFs results from increased Ca²⁺ release from internal stores**

Our previous findings that Cdk5 regulates mitochondrial Ca²⁺ concentration ([Ca²⁺]mt) by controlling ER Ca²⁺ transfer to the mitochondria [34] led us to investigate whether Cdk5 also regulates free cytosolic Ca²⁺ concentration ([Ca²⁺]cyt). To do so, we initially performed spectrofluorometric analysis to measure resting [Ca²⁺]cyt in Fluo-4 AM-loaded primary MEFs [34] isolated from wt and Cdk5−/− mice embryos (Fig. 1A). As shown in Fig. 1B, the resting [Ca²⁺]cyt in Cdk5−/− MEFs was higher (p < 0.01) compared to that in wt MEFs (150 vs 100 nM), suggesting that loss of Cdk5 elicits a rise in free [Ca²⁺]cyt. We then examined three possible mechanisms that may have caused the rise in [Ca²⁺]cyt in Cdk5−/− MEFs: (i) reduced internal Ca²⁺ store capacity, (ii) increased Ca²⁺ influx from the extracellular milieu, and (iii) increased Ca²⁺ release from internal Ca²⁺ stores. We began by treating wt and Cdk5−/− MEFs with thapsigargin (TG), a potent non-competitive inhibitor of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) pump [45], that empties internal Ca²⁺ stores, allowing measurement of internal Ca²⁺ store capacity. This was followed by subjecting cells to external 2 mM CaCl₂ to measure capacitative Ca²⁺ entry from the extracellular milieu. As
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Fig. 1 Cdk5\(^{-/-}\) MEFs exhibit increased \([Ca^{2+}]_{cyt}\) and increased ATP-induced Ca\(^{2+}\) release from internal stores. **A** Lysates of MEFs isolated from wt and Cdk5\(^{-/-}\) mouse embryos were analyzed by SDS-PAGE and immunoblotting for Cdk5. Actin blot was used as loading control. **B** Cdk5 loss caused an increase in free \([Ca^{2+}]_{cyt}\). Wt and Cdk5\(^{-/-}\) MEFs loaded with the cell-permeable intracellular Ca\(^{2+}\) indicator, Fluo-4 AM, were analyzed for \([Ca^{2+}]_{cyt}\) using spectrofluorometric Ca\(^{2+}\) imaging. Free \([Ca^{2+}]_{cyt}\) levels were measured as described in Materials and methods. Data represent means ± SEM from six independent experiments (n = 6). * indicates p < 0.01. **C** Wt and Cdk5\(^{-/-}\) MEFs loaded with Fluo-4 AM were analyzed for \([Ca^{2+}]_{cyt}\) transients following the addition of TG (1 µM) by single-cell Ca\(^{2+}\) imaging as described in Materials and methods. TG-induced Ca\(^{2+}\) release from internal stores corresponds to internal Ca\(^{2+}\) store capacity. After initial analysis in [Ca\(^{2+}\)]-free buffer, capacitative Ca\(^{2+}\) entry from the extracellular milieu was determined upon addition of 2 mM CaCl\(_2\) in the presence of TG. Data represent means of Ca\(^{2+}\) signal traces from 15 cells. **D** and **E** Loss of Cdk5 caused an increase in ATP-induced Ca\(^{2+}\) release from internal stores. \([Ca^{2+}]_{cyt}\) in wt and Cdk5\(^{-/-}\) MEFs was measured following stimulation with 0.1 µM (D) or 1 µM (E) ATP in [Ca\(^{2+}\)]-free buffer by single-cell Ca\(^{2+}\) imaging. Graphs in (D) and (E), right panels, represent peak amplitude values and integrated Ca\(^{2+}\) signals, which is the area under the curve (AUC area that begins immediately after addition of 0.1 mM ATP and ends when the Ca\(^{2+}\) trace goes back to the baseline level). Data represent means of Ca\(^{2+}\) signal traces from 20 cells. All values are means ± SEM from three independent experiments (n = 3). *p < 0.05
shown in Fig. 1C, there was no difference in internal Ca\(^{2+}\) store capacity and capacitative external Ca\(^{2+}\) entry in wt and Cdk5\(^{-/-}\) MEFs. Since ATP, which binds cell surface purinergic receptors [46], induces Ca\(^{2+}\) release from internal stores [47] in Ca\(^{2+}\)-free buffer, we examined whether increased [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs is due to internal store Ca\(^{2+}\) release by loading cells with Fluo-4 AM and treating them with ATP in Ca\(^{2+}\)-free EDTA-containing buffer. By single-cell Ca\(^{2+}\) imaging analyses using a confocal laser scanning microscope, we found that 0.1 µM (Fig. 1D) and 1 µM (Fig. 1E) ATP caused greater [Ca\(^{2+}\)]\(_{cyst}\) transients in Cdk5\(^{-/-}\) MEFs compared to wt, with greater rise in [Ca\(^{2+}\)]\(_{cyst}\) transient at 1 µM ATP. These findings suggest that elevated ATP-evoked rise in [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs results from increased Ca\(^{2+}\) release from internal stores.

**Elevated ATP-evoked rise in [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs is due to increased Ca\(^{2+}\) release via IP3R channels**

We next tested whether the ATP-induced rise in [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs occurs through IP3R. As shown in Fig. 2A, treatment of Fluo-4 AM-loaded and ATP-stimulated MEFs with xestospongin C (XeC) [48], a potent IP3R inhibitor, caused complete inhibition of the ATP-evoked [Ca\(^{2+}\)]\(_{cyst}\) increase in both wt and Cdk5\(^{-/-}\) MEFs, indicating that such [Ca\(^{2+}\)]\(_{cyst}\) increase is mediated by IP3R, which forms Ca\(^{2+}\) channels in the internal Ca\(^{2+}\) stores [47]. To examine whether loss of Cdk5 alters the IP3-mediated Ca\(^{2+}\) release from the ER, cells loaded with the ER Ca\(^{2+}\) probe, Mag-Fluo-4 AM [49], were treated with IP3 after permeabilization with saponin to facilitate IP3 access to IP3R. By single-cell Ca\(^{2+}\) imaging, we found that IP3 induced a greater decline in Mag-Fluo-4 signal in Cdk5\(^{-/-}\) MEFs compared to wt (Fig. 2B). Together, these results indicate that elevated ATP-evoked rise in [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs is due, at least in part, to increased ER Ca\(^{2+}\) release through IP3R channels.

**Cdk5 interaction with and phosphorylation of IP3R1 at S\(_{421}\) downregulate IP3R1-mediated ER Ca\(^{2+}\) release**

We then sought to further investigate how ATP-evoked IP3R-mediated ER Ca\(^{2+}\) release increases in Cdk5\(^{-/-}\) MEFs occurs through IP3R. As shown in Fig. 3A, treatment of Fluo-4 AM-loaded and ATP-stimulated MEFs with xestospongin C (XeC) [48], a potent IP3R inhibitor, caused complete inhibition of the ATP-evoked [Ca\(^{2+}\)]\(_{cyst}\) increase in both wt and Cdk5\(^{-/-}\) MEFs, indicating that such [Ca\(^{2+}\)]\(_{cyst}\) increase is mediated by IP3R, which forms Ca\(^{2+}\) channels in the internal Ca\(^{2+}\) stores [47]. To examine whether loss of Cdk5 alters the IP3-mediated Ca\(^{2+}\) release from the ER, cells loaded with the ER Ca\(^{2+}\) probe, Mag-Fluo-4 AM [49], were treated with IP3 after permeabilization with saponin to facilitate IP3 access to IP3R. By single-cell Ca\(^{2+}\) imaging, we found that IP3 induced a greater decline in Mag-Fluo-4 signal in Cdk5\(^{-/-}\) MEFs compared to wt (Fig. 2B). Together, these results indicate that elevated ATP-evoked rise in [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs is due, at least in part, to increased ER Ca\(^{2+}\) release through IP3R channels.

**Fig. 2** Increased [Ca\(^{2+}\)]\(_{cyst}\) in Cdk5\(^{-/-}\) MEFs is due to increased IP3R-mediated Ca\(^{2+}\) release. A MEFs loaded with Fluo-4 AM and treated with 3 µM XeC followed by 1 µM ATP then 2 µM TG in Ca\(^{2+}\)-free EGTA-containing KRH buffer were analyzed for [Ca\(^{2+}\)]\(_{cyst}\) transients by single-cell Ca\(^{2+}\) imaging analyses. The similar increase in [Ca\(^{2+}\)]\(_{cyst}\) in wt and Cdk5\(^{-/-}\) MEFs upon treatment with TG, which was added after 15 min of treatment with XeC, indicates comparable viability of these cells during analysis. Data represent means of Ca\(^{2+}\) signal traces from 15 cells. B Measurement of Ca\(^{2+}\) release from internal stores upon IP3 treatment is described in Materials and methods. Ca\(^{2+}\) release was measured every 4 s by single-cell Ca\(^{2+}\) imaging. Data represent means of Ca\(^{2+}\) signal traces from ten cells, and are results from one of three independent experiments showing similar patterns. Values are means ± SEM from the three separate experiments (n = 3). *p < 0.05

for Cdk5. Co-IP of Cdk5 with IP3R1 (Fig. 3A) indicates interaction between the two proteins. However, co-transfection of Cdk5, p35 and IP3R1 in HEK293T cells followed by immunoprecipitation of Cdk5 or p35 showed the presence of the Cdk5/p35/IP3R1 complex. However, co-transfection of Cdk5 and IP3R1, but not p35, also showed Cdk5 interaction with IP3R1, indicating that such interaction does not require p35 (Supplementary Fig. 3C). We then examined whether Cdk5 phosphorylates its potential targets in IP3R1, S\(_{421}\)PKL and T\(_{799}\)PVK, by analyzing lysates of wt and Cdk5\(^{-/-}\) MEFs. Immunoblotting showed that while there was no difference in IP3R1 Thr\(_{799}\) phosphorylation in wt and Cdk5\(^{-/-}\) MEFs, IP3R1 Ser\(_{421}\) phosphorylation was reduced (p < 0.05) in Cdk5\(^{-/-}\) MEFs compared to wt (Fig. 3B), indicating that Cdk5 specifically targets Ser\(_{421}\) in IP3R1. The inability of IP3R1 Ser\(_{421}\) antibody to detect non-phosphorylatable IP3R1 S\(_{421}\)A (Fig. 3C) confirms the specificity of the antibody.
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Fig. 3 Cdk5 associates with and phosphorylates IP3R1 at Ser\(_{421}\). A Cdk5 associates with IP3R1. Lysates of wt and Cdk5\(^{-/-}\) MEFs were subjected to immunoprecipitation (IP) using IP3R1 antibody. The IPs were resolved by 4–20% gradient SDS-PAGE and then immunoblotted for IP3R1 and Cdk5 (left panel). To assess the specificity of the IP3R1 antibody, lysates of wt MEFs were blotted (right panel) with antibody blocked (lane 2) or not blocked (lane 1) with the peptide antigen that was used to raise the antibody. Lanes 3 and 4 represent IP control using normal IgG.

B Cdk5 specifically phosphorylates IP3R1 at Ser\(_{421}\). Lysates of wt and Cdk5\(^{-/-}\) MEFs were subjected to SDS-PAGE and then immunoblotted for IP3R1 phosphoSer\(_{421}\) and phosphoThr\(_{799}\), IP3R1, Cdk5 and tubulin. Tubulin blot was used as loading control. Representative blots are from one of four independent experiments (\(n=4\)) showing similar results. Ratios of levels of IP3R1 phosphoSer\(_{421}\) (middle panel) and phosphoThr\(_{799}\) (right panel) vs total IP3R were calculated following densitometric analysis of blots using NIH Image J 1.61. Standard deviations were calculated based on the ratios obtained from the four independent sets of experiments. Values from wt MEFs were normalized to 1.0. *\(p<0.05\); ns: not significant.

C Shows the specificity of the IP3R1 phosphoSer\(_{421}\) antibody. Lysates of wt MEFs depleted of endogenous IP3R1, but expressing exogenous IP3R1 S\(_{421}\)A (res) were subjected to immunoblotting for IP3R1 phosphoSer\(_{421}\), IP3R1, and Cdk5. GAPDH blot was used as loading control.
Fig. 4 IP3R1 loss inhibits the ATP-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in Cdk5−/− MEFs. A Lysates of cells transfected with IP3R1 siRNA #1 or #2 for 48 h were resolved by SDS-PAGE and immunoblotting for IP3R1 and Cdk5. Tubulin blot was used to assess protein loading. Representative blots are from one of three independent experiments showing similar result are shown. B wt and Cdk5−/− MEFs transfected with IP3R1 siRNA #1 or #2, loaded with Fluo-4 AM, and treated with 1 µM ATP were analyzed for \([\text{Ca}^{2+}]_{\text{cyt}}\), transients by single-cell Ca2+ imaging analyses in Ca2+-free buffer. Data are means of Ca2+ signal traces from 20 cells and are from one of three independent experiments showing similar results. [Ca2+]cyt transients were further analyzed by measuring their peak amplitudes (C) and calculating the areas under the curve which begin immediately after addition of 1 mM ATP and ends when the Ca2+ trace goes back to the baseline level. D Values are means ± SEM from three independent experiments (n=3). * and ** Denote p<0.05 and p<0.01, respectively. E wt and Cdk5−/− MEFs were co-transfected with the indicated vector and IP3R1 siRNA #2. Cell lysates (40 µg) were resolved by SDS-PAGE and immunoblotted for IP3R1 and Cdk5. GAPDH blot was used as loading control. F wt and Cdk5−/− MEFs co-transfected with the indicated vector and siRNA #2 were loaded with Mag-Fluo-4 AM. ER Ca2+ release following IP3 treatment was measured by spectrofluorometry. Values, which represent the fold change in peak amplitudes, are means ± SEM from three independent experiments (n=3). *p<0.05

Fig. 4E, Cdk5−/− MEFs transfected with empty pcDNA3.0 displayed greater IP3-induced Ca2+ release than wt MEFs, which is consistent with our data in Fig. 2B. Expression of exogenous IP3R1 S421D caused complete inhibition of IP3-induced ER Ca2+ release in both wt and Cdk5−/− MEFs depleted of endogenous IP3R1, while expression of exogenous IP3R1 S421A caused further increase in ER Ca2+ release compared to control vector-transfected cells. These findings and our earlier data, showing that Cdk5 loss, which reduces inhibitory phosphorylation of IP3R1 S421 (Fig. 3B), causes elevated IP3-induced Ca2+ release suggest that Cdk5 serves to downregulate ER Ca2+ release through inhibitory phosphorylation of IP3R1 at S421.

Increased \([\text{Ca}^{2+}]_{\text{cyt}}\) due to Cdk5 loss induces ROS production, which upregulates Nrf2 level

Since dysregulated \([\text{Ca}^{2+}]_{\text{cyt}}\) homeostasis affects intracellular ROS level [50, 51], and loss of Cdk5 alters \([\text{Ca}^{2+}]_{\text{cyt}}\), we next sought to assess intracellular ROS levels in wt and Cdk5−/− MEFs. Cells stained with a fluorescent cytosolic ROS probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), were analyzed for intracellular hydrogen peroxide level by live-cell imaging using an Olympus IX71 fluorescent microscope. As shown in Fig. 5A (left panel), Cdk5−/− MEFs displayed increased DCFDA staining compared to wt. Consistent with the microscopic data, flow cytometry analysis showed an increase (p<0.05) in intracellular hydrogen peroxide level in Cdk5−/− MEFs compared to wt MEFs (Fig. 5A, right panel). Since mitochondria are a major source of ROS, we also examined ROS levels in this organelle by MitoSOX staining. Figure 5B (left panel) shows that mitochondrial superoxide anion levels were likewise higher in Cdk5−/− MEFs compared with wt. Similarly, flow cytometry analysis showed an increase in mitochondrial superoxide anions in Cdk5−/− MEFs compared to wt (Fig. 5B, right panel). These observations indicate that loss of Cdk5 induces ROS production.

ROS tightly regulates the activity of nuclear factor erythroid 2-related factor 2 (Nrf2) [52], which responds to oxidative stress by binding to the antioxidant response element (ARE) in the promoter of genes coding for antioxidant enzymes such as peroxiredoxin 1 and 2 (Prx1 and Prx2). Thus, we further examined the levels of Nrf2 in wt and Cdk5−/− MEFs. Interestingly, Cdk5 loss, which induces ROS production, upregulates Nrf2 level as well as levels of the Nrf2 antioxidant protein targets, Prx1 and Prx2 (Fig. 6A). To establish a link between increased ROS level and upregulated Nrf2 level in Cdk5−/− MEFs, cells treated with a ROS scavenger, mito-tempo or reduced glutathione (GSH) were (i) stained with DCFDA and analyzed for cytoplasmic ROS level by live-cell imaging, and (ii) subjected to SDS-PAGE and immunoblotting for Nrf2. As shown in Fig. 6B, and
C, mito-tempo and GSH prevented the increase in ROS (Fig. 6B) and Nrf2 (Fig. 6C) levels in Cdk5−/− MEFs. ROS and Nrf2 levels in these cells were reduced by the ROS scavengers to a level equivalent to that in untreated wt MEFs, indicating that increased ROS level in Cdk5−/− MEFs upregulates Nrf2 level in these cells.

Our next step was to examine the effect of Cdk5-regulated [Ca2+]cyt on ROS level. To do so, wt and Cdk5−/− MEFs treated with XeC, ionomycin or BAPTA-AM and then stained with DCFDA (Fig. 7A) or mitoSOX red (Fig. 7B) were subjected to flow cytometry. As shown in Fig. 7A and B, inhibition of IP3R-mediated Ca2+ release with XeC and chelating Ca2+ with BAPTA-AM in Cdk5−/− MEFs reduced ROS production to a level close to that in wt. As expected, treatment of Cdk5−/− MEFs with the membrane permeable Ca2+ ionophore, ionomycin, increased ROS level. These findings indicate that increased [Ca2+]cyt in Cdk5−/− MEFs upregulates ROS production. We then examined the effect of scavenging ROS with GSH or mito-tempo on [Ca2+]cyt in Cdk5−/− MEFs. To do so, wt and Cdk5−/− MEFs treated with GSH or mito-tempo were stained with Fluo-4 AM to measure [Ca2+]cyt in these cells. As shown in Supplementary Fig. 4, GSH and mito-tempo had no effect on [Ca2+]cyt in Cdk5−/− MEFs under a condition where BAPTA-AM reduced [Ca2+]cyt to a level similar to that in wt. These results indicate that while [Ca2+]cyt regulates ROS production in Cdk5−/− MEFs, ROS level does not influence [Ca2+]cyt in these cells.

**Altered Ca2+ dynamics in Cdk5−/− MEFs correspond to accelerated cell proliferation that correlates with increased body weight and size in Cdk5−/− embryos**

IP3R-mediated Ca2+ transients regulate G1/S transition during cell-cycle progression [53] and cell proliferation [18, 19], and Cdk5, which regulates intracellular Ca2+ dynamics, has been implicated in cell proliferation [7, 8]. This prompted us to test whether altered Ca2+ dynamics in ex vivo Cdk5−/− MEFs is associated with proliferation defect. As shown in Fig. 8A, Cdk5−/− MEFs proliferate at a faster rate compared to wt, indicating that Cdk5 loss, which triggers a rise in [Ca2+]cyt, accelerates proliferation in MEFs. To establish a link between altered Ca2+ dynamics and increased proliferation in Cdk5−/− MEFs, proliferation of cells treated with XeC was examined. As shown in Fig. 8A, treatment with XeC reversed the increase in Cdk5−/− MEF proliferation to a level equivalent to that in wt, indicating that accelerated proliferation
Cdk5 regulates IP3R1-mediated Ca2+ dynamics and Ca2+-mediated cell proliferation

in Cdk5−/− MEFs is linked to altered IP3R-mediated Ca2+ dynamics. We then tested whether the proliferation error in Cdk5−/− MEFs is recapitulated in mice. Since Cdk5−/− mice exhibit perinatal mortality (i.e., 64% die in utero and newborns are either dead or weak and die within 12 h after birth) [54], we isolated embryonic day 16.5 (E16.5) Cdk5+/+ and Cdk5−/− embryos (Fig. 8B) from pregnant Cdk5+/− mice, and body weights and sizes were compared. As shown in Fig. 8C, the Cdk5−/− embryos weighed more (p < 0.05) than their wt littermates. Figure 8D shows representative wt and Cdk5−/− littermate embryos with the Cdk5−/− embryo clearly bigger than the wt. By immunohistochemistry, we found increased staining for Ki67, a proliferation marker, in the prefrontal cortex, olfactory epithelium, lung, and duodenum of Cdk5−/− embryos compared to wt (Fig. 8E), which likely accounts for their increased body weight and size.

Discussion

Our previous finding that Cdk5 localizes in MAMs, and regulates [Ca2+]mt by controlling ER Ca2+ transfer to the mitochondria [34], points to a role for Cdk5 in regulating intracellular Ca2+ dynamics. However, the involvement of Cdk5 in this process remains to be investigated. In this study, using Cdk5−/− MEFs, we demonstrated that the IP3R1 Ca2+ channel is a downstream target of Cdk5, which interacts with and phosphorylates IP3R1 at Ser421, a target that lies in the IP3-binding site. As illustrated in our proposed model (Fig. 9), Cdk5 phosphorylation of IP3R1 Ser421 controls IP3R1-mediated internal Ca2+ release as loss of Cdk5 in MEFs, and thus, loss of IP3R1 Ser421 phosphorylation triggers an increase in IP3R1-mediated Ca2+ release from internal stores, resulting in elevated [Ca2+]cyt. This rise in [Ca2+]cyt causes accelerated proliferation in Cdk5−/− MEFs.
which correlates with increased body weight and size in Cdk5−/− mouse embryos.

While the rise in free [Ca^{2+}]_{cyt} in Cdk5−/− MEFs could be due to reduced internal Ca^{2+} store capacity or increased Ca^{2+} influx from the extracellular milieu, we did not observe either of these in Cdk5−/− MEFs. To test the possibility that loss of Cdk5 perturbs Ca^{2+} release from the ER to cause increased [Ca^{2+}]_{cyt}, we took advantage of the fact that ATP mediates Ca^{2+} release from internal stores [47], and that an ER Ca^{2+} probe, Mag-Fluo-4 AM, may be utilized to
Fig. 8 Cdk5−/− MEFs show increased proliferation and Cdk5−/− mouse embryos are heavier and bigger than wt. A Cdk5−/− MEFs proliferate at a faster rate compared to wt, but treatment with XeC reverses this phenotype in Cdk5−/− MEFs. Proliferation was measured as described in Materials and methods. B Homogenates (20 μg) of tails from E16.5 wt and Cdk5−/− embryos were analyzed by SDS-PAGE and immunoblotting for Cdk5. Actin blot was used as loading control. C Body weights of E16.5 wt (n = 4) and Cdk5−/− (n = 5) embryos from four litters were measured. *p < 0.05. D Representative images of E16.5 wt and Cdk5−/− littermates. E Immunohistochemistry of prefrontal cortex, olfactory epithelium, lung and duodenum from wt and Cdk5−/− littermates. Embryos were sectioned to 10 μm thickness and stained for Ki67. Arrows are directed at Ki67-positive cells. The graph showing the percentage of Ki67-positive cells was calculated from five non-overlapping fields (n = 5). *p < 0.05
Ca²⁺

ER

IP3R1

Cdk5

↓ [Ca²⁺]ₙₚ

Cell proliferation ↑

↓ ROS

↑ Nrf2

Antioxidant enzymes

Fig. 9 Proposed model illustrating how Cdk5 phosphorylation of IP3R1 (Ser₄₂₁) controls IP3R1-mediated internal Ca²⁺ release and [Ca²⁺]ₙₚ (green text and arrow) and how loss of Cdk5 in Cdk5⁻/⁻ MEFs affects [Ca²⁺]ₙₚ and Ca²⁺-mediated processes (black text and arrows). Loss of Cdk5 reduces the phosphorylation of IP3R1 Ser₄₂₁, causing increased IP3R1-mediated Ca²⁺ release. Subsequent rise in [Ca²⁺]ₙₚ increases ROS production, causing increased Nrf2 expression and activity, and increased expression of the NRF2 antioxidant targets such as Prx1 and Prx2. Adequate [Ca²⁺]ₙₚ permits progression of Ca²⁺-mediated proliferation, but excess levels cause increased cell proliferation.

Measure ER Ca²⁺ release. Using this approach, we found greater ATP-induced ER Ca²⁺ release in Cdk5⁻/⁻ MEFs compared to wt. IP3R inhibition with XeC [55] completely blocks this ATP-evoked [Ca²⁺]ₙₚ increase in both wt and Cdk5⁻/⁻ MEFs, indicating that such [Ca²⁺]ₙₚ increase is mediated by the IP3R Ca²⁺ channels in the ER. Our finding that IP3 induces a greater decline in Mag-Fluo-4 signal in Cdk5⁻/⁻ MEFs compared to wt further supports our view that Cdk5 serves to control the IP3R-mediated ER Ca²⁺ release that leads to increased [Ca²⁺]ₙₚ in Cdk5⁻/⁻ MEFs.

Since IP3R-mediated Ca²⁺ release is regulated by IP3R phosphorylation [18], and Cdk5, which localizes in MAMs [34], has two potential phosphorylation target sites in IP3R1, S₄₂₁, PLK, and T₉₉₉PVK, that exist in the IP3-binding site, we examined whether Cdk5 interacts with and phosphorylates IP3R1. Indeed, we found that Cdk5 interacts with and specifically phosphorylates IP3R1 at Ser₄₂₁. This was demonstrated by reduced IP3R1 Ser₄₂₁ phosphorylation in Cdk5⁻/⁻ MEFs, which increases in ER Ca²⁺ release. The specificity of IP3R1 immunoreactivity was verified by the loss of detectable IP3R1 in wt MEFs when the IP3R1 antibody was blocked with the peptide antigen that was used to generate the antibody. The detection of two IP3R1 immunoreactive bands may reflect immunoreactivity with (i) both unphosphorylated and phosphorylated forms, (ii) different isoforms, or (iii) intact and degraded forms of the protein. Partial reduction (p < 0.05) of IP3R1 S₄₂₁ phosphorylation in Cdk5⁻/⁻ MEFs compared to wt suggests the presence of at least one other IP3R1 S₄₂₁ kinase. In fact, Cdk1 has been shown to phosphorylate IP3R1 S₄₂₁ [37]. Although IP3R1 S₄₂₁A substitution was shown to increase IP3 binding to IP3R1 [35], its effect on ER Ca²⁺ release has not been investigated. Our data show that in endogenous IP3R1-depleted cells, exogenous IP3R1 S₄₂₁D (res) expression inhibits ER Ca²⁺ release, while IP3R1 S₄₂₁A expression enhances ER Ca²⁺ release substantiate the importance of inhibitory phosphorylation of IP3R1 S₄₂₁, which prevents Ca²⁺ release from internal stores. Apparently, Cdk5 plays a significant role in this process. Our findings support the idea that MAM-associated Cdk5 negatively regulates the opening of the IP3R Ca²⁺ channel through phosphorylation of IP3R1 Ser₄₂₁, which, as indicated above, lies in the IP3-binding site. It is interesting that ERK1/2 phosphorylation of Ser₄₃₆, which also lies in the IP3-binding site, inhibits the opening of the IP3R channel as well [39, 40]. Since a rise in [Ca²⁺]ₙₚ can activate Ca²⁺-induced Ca²⁺ release (CICR) [56] from the internal stores, elevated ER Ca²⁺ release in Cdk5⁻/⁻ MEFs may propagate Ca²⁺ signals to neighboring organelles, causing a further rise in [Ca²⁺]ₙₚ.

In addition to triggering a rise in free [Ca²⁺]ₙₚ, loss of Cdk5 in MEFs further induces ROS production. The ability of XeC and BAPTA-AM to reverse the increase in ROS level in Cdk5⁻/⁻ MEFs indicates that ROS production occurs downstream of the IP3R1-mediated increase in [Ca²⁺]ₙₚ in these cells. Interestingly, we found that increased ROS production in Cdk5⁻/⁻ MEFs is associated with increased proliferation. Since increased ROS also induces apoptosis, it is possible that ROS-associated upregulation of Nrf2 and its antioxidant protein targets, Prx1 and Prx2, in Cdk5⁻/⁻ MEFs acts in a feedback control loop, ensuring that the ROS level in these cells does not exceed the threshold level that triggers apoptosis. This notion indicates the adaptability of MEFs under increased oxidative stress condition.

Cdk5 and IP3R-mediated Ca²⁺ oscillations have been shown to regulate cell-cycle progression [6, 7, 10, 11, 53] and proliferation [12–19]. Thus, it is not surprising that Cdk5⁻/⁻ MEFs, which have elevated [Ca²⁺]ₙₚ through IP3R1, proliferate at a faster rate compared to wt. The ability of XeC to reverse the increase in Cdk5⁻/⁻ MEF proliferation to a level equivalent to that in wt supports our view that accelerated proliferation in Cdk5⁻/⁻ MEFs is linked to IP3R-mediated increase in [Ca²⁺]ₙₚ. This is consistent with previous reports that Cdk5 plays an inhibitory role in the neuronal cell cycle [6, 10, 11]. In addition, reduced level of p27Kip1 in Cdk5⁻/⁻ MEFs is consistent with the fact that IP3R-mediated Ca²⁺ oscillations stimulate proliferation through downregulation of p27Kip1 [1, 53]. Proliferation error in Cdk5⁻/⁻ MEFs correlates with increased weight and...
size in E16.5 Cdk5−/− embryos, and increased number of Ki67-positive cells in various embryonic tissues, including prefrontal cortex, olfactory epithelium, lung and duodenum. Although we observed the same trend in body weight and size in earlier E13.5 Cdk5−/− embryos, we note that later E18.5 Cdk5−/− embryos were lighter and smaller than their wt counterparts. This may be due to the development of other abnormalities in Cdk5−/− embryos as they exhibit perinatal mortality. It is known that ~64% of Cdk5−/− embryos die in utero and newborns are either dead or weak and die within 12 h after birth [54]. Nonetheless, increased body weight and size in E16.5 Cdk5−/− embryos are consistent with reduced levels of the cell-cycle inhibitors, p21CIP1 and p27KIP1, and increased body weight in p27KIP1 knockout mice [57].

In summary, we provide evidence that Cdk5 controls intracellular Ca2+ dynamics through phosphorylation of IP3R1 at Ser421, and Ca2+-mediated cell proliferation as indicated by increased Cdk5−/− MEF proliferation that correlates with increased body weight and size in Cdk5−/− embryos.

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Author contributions SN performed the experiments for Figs. 1–5, 7, 8A, 9 and Supplementary Figs. 1, 2, 3 and 4, and wrote a draft of the manuscript. VL performed the experiments for Fig. 8B–E and Supplementary Fig. 5. JL performed the experiments for Fig. 6. KYL and JLR contributed to the analysis and interpretation of data and/or experimental design, critically revised the manuscript for important intellectual content, and wrote the final version of the manuscript.

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

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval All studies involving mice, which were maintained at the University of Calgary Animal Facility, conformed to regulatory standards and were approved by the University of Calgary Health Sciences Animal Care Committee.

Consent to participate Not applicable.

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