

TRPM7 regulates quiescent/proliferative metabolic transitions in lymphocytes

Jaya Sahni,^{1,3} Richard Tamura,² Ian R. Sweet² and Andrew M. Scharenberg^{1,3,*}

¹Department of Pediatrics and Immunology; ²Diabetes and Obesity Center of Excellence; Department of Medicine; University of Washington;

³Seattle Children's Research Institute; Seattle, WA USA

Key words: aerobic glycolysis, lymphocyte, metabolism, quiescence, TRPM7

A unique property of lymphocytes among all body tissues is their capacity for rapid proliferation in the context of responding to infectious challenges. Lymphocyte proliferation involves a transition from a quiescent metabolic state adjusted to maintain cellular energy homeostasis, to a proliferative metabolic state in which aerobic glycolysis is used to generate energy and biosynthetic precursors necessary for the accumulation of cell mass. Here we show that modulation of TRPM7 channel function in tumor B lymphocytes directly induces quiescent/proliferative metabolic transitions. As TRPM7 is widely expressed outside of the immune system, our results suggest that TRPM7 may play an active role in regulating metabolic transitions associated with rapid cellular proliferation and malignancy.

Introduction

Lymphocyte expansion is regulated through proliferative signals received through multiple types of receptors. When the “ensemble” signal a lymphocyte receives reaches a threshold sufficient to drive proliferation, lymphocytes radically shift their metabolism from a state of quiescence adjusted to support cellular energy homeostasis, to a state of anabolism that supports a rapid accumulation of cell mass and completion of one or more cell cycles. When the “ensemble signal” falls below the proliferative threshold, metabolism returns to its original quiescent state, mass accumulation ceases and the lymphocyte exits the cell cycle. A key regulator of these quiescent/proliferative transitions is the PI3K/Akt/mTOR pathway, which directly controls cellular energy metabolism and catabolism/anabolism balance in response to the availability of nutrients and myriad types of growth-promoting hormones.

The dual function ion channel-protein kinase–transient receptor potential cation channel, subfamily M, member 7 (TRPM7) has recently been shown to support sustained phosphoinositide 3-kinase (PI3K) signaling in proliferating lymphocytes,¹ positioning TRPM7 alongside PI3K as an important component in the processes which control lymphocyte metabolism. TRPM7's influence on lymphocyte metabolism is linked to its role in regulation of cellular Mg²⁺ uptake: TRPM7-deficient cells downregulate PI3K/Akt/mTOR signaling and undergo proliferative arrest when transitioned to standard tissue culture media; this phenotype is suppressible either by provision of supplemental extracellular Mg²⁺ or heterologous expression of a Mg²⁺ transporter.^{2,3}

A striking aspect of the proliferative arrest exhibited by TRPM7-deficient cells is that a high percentage of arrested cells are positioned at the beginning of the cell cycle as indicated by flow cytometric analysis of DNA content and cell size.¹ Similar DNA content and size parameters are observed in primary lymphocytes positioned in quiescence/G₀,^{4,5} suggesting the potential involvement of TRPM7 in processes required to transition out of quiescence/G₀. In this study, we examined the biological events that occur when TRPM7-deficient lymphocytes are proliferation-arrested in standard tissue culture media and observed that these cells exhibit multiple signatures associated with quiescence. Provision of either supplemental Mg²⁺ or TRPM7-mediated Mg²⁺ entry fully reversed the key features associated with quiescence, implicating TRPM7-mediated Mg²⁺ uptake as a key mechanism required for cell cycle re-entry from G₀.

Results

TRPM7-deficient B cells reversibly exit cell cycle and enter quiescence. We have previously shown that TRPM7-deficient cells stop accumulating mass and cease to proliferate when moved from Mg²⁺ supplemented media to regular media.¹ In order to better understand the nature of the metabolic transition that occurs when these cells are shifted from supplemental to physiological external Mg²⁺, we compared WT cells cultured in standard tissue culture/regular media, TRPM7-KO (or TRPM7-deficient) cells in growth-supporting media (media with 15 mM Mg²⁺; +media) and TRPM7-deficient cells transitioned to regular media for 24–48 hrs, which were labeled with CFSE (Fig. 1). When a CFSE labeled cell enters the cell cycle, each daughter

*Correspondence to: Andrew M. Scharenberg; Email: andrewms@u.washington.edu

Submitted: 05/10/10; Revised: 06/25/10; Accepted: 06/25/10

Previously published online: www.landesbioscience.com/journals/cc/article/12798

DOI: 10.4161/cc.9.17.12798

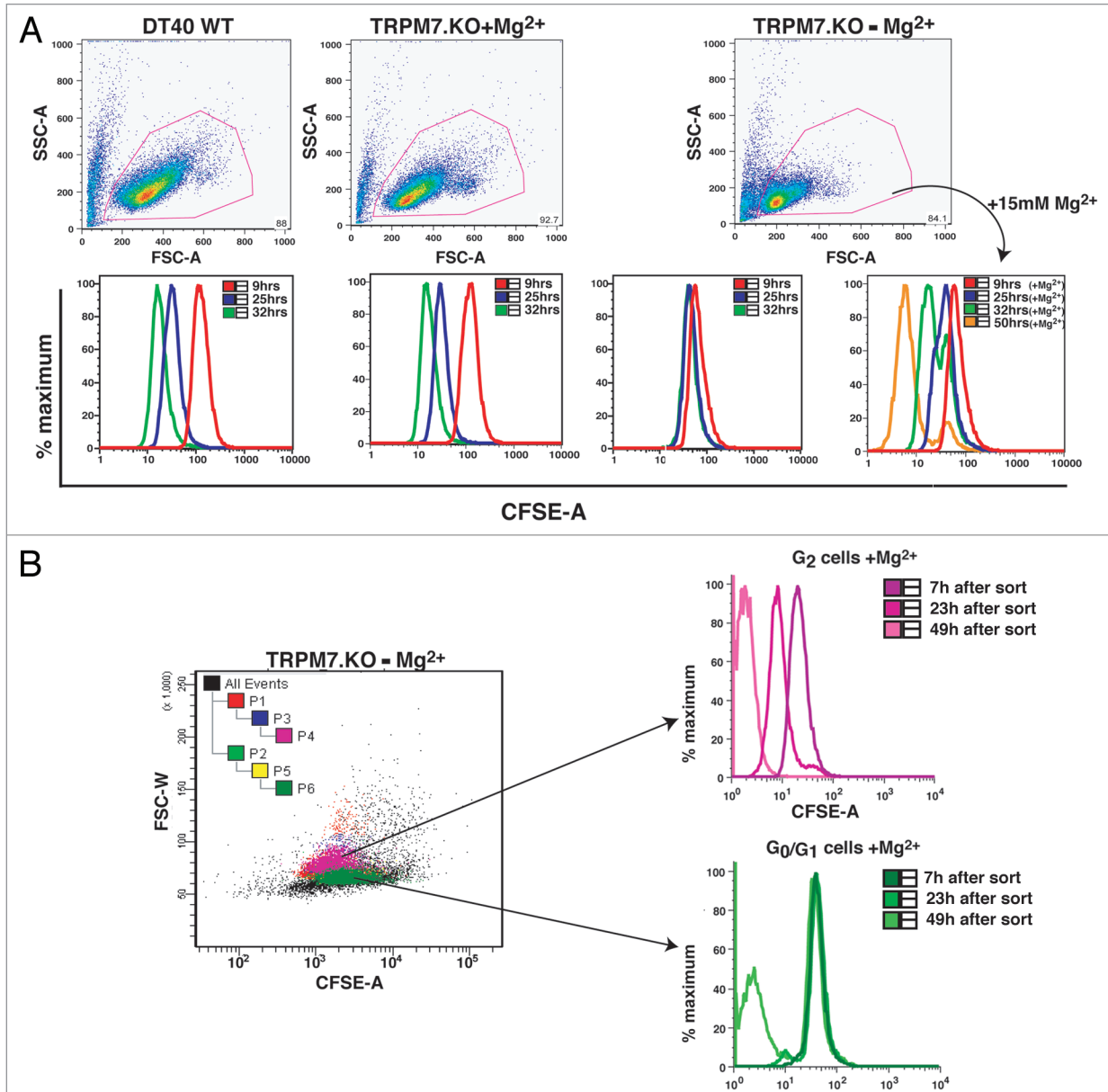


Figure 1. Proliferation arrested TRPM7-KO cells exhibit stable CFSE staining upon transition to regular media. (A) TRPM7-deficient cells transitioned to regular media for 24 hours undergo proliferative arrest and show stable CFSE staining. Top part: Forward scatter vs. side scatter analysis displaying cell size distribution of WT cells in regular media, TRPM7-deficient cells in growth-supporting media with supplemental Mg²⁺ and TRPM7-KO cells transitioned to regular media for 24 hours without and with re-addition of 15 mM Mg²⁺. All cell lines were labeled with CFSE 24 hrs post transition of TRPM7-KO cells to regular media. Data was acquired on BD LSRII flow cytometer and analyzed by Flowjo (Tree Star, Inc.; Ashland, Oregon). Bottom part: CFSE-labeled cells were acquired at 9, 24 and 32 hours and analyzed by Flowjo. By 25 hours (time not including 24 hours post transition), TRPM7-deficient cells stop undergoing further rounds of proliferation in regular media (-media) without 15 mM supplemental Mg²⁺ and display substantially stable CFSE staining (3rd bottom part). Provision of supplemental Mg²⁺ at 24 hrs post transition allowed recovery of only a small proportion of cells by 32 hours (4th bottom part). (B) Proliferation-arrested TRPM7-KO cells resume cell division in presence of supplemental Mg²⁺. TRPM7-deficient cells cultured in regular media for 24 hours were labeled with CFSE and sorted on the basis of their cell size (smaller G₀/G₁ and larger G₂ cells; see Suppl. Fig.1) followed by provision of 15 mM supplemental Mg²⁺ to both populations. Further reacquisition of these two populations at 7, 23 and 49 hours indicated that both G₁ and G₂ cells are able to resume cell division.

cell undergoes a 50% decrease in CFSE fluorescence as the dye is partitioned between daughter cells at each cell division; if all cells of a population are able to continue rapid division, a progressive decrease in population CFSE fluorescence is observed.⁶ Our analyses show that both WT cells growing in regular media and TRPM7-KO cells in growth-supporting media continue

their rapid proliferation and exhibit a progressive loss of CFSE fluorescence over time in culture. In contrast, TRPM7-KO cells transitioned to regular media exhibit nearly completely stable CFSE staining (Fig. 1A), consistent with their proliferative arrest in a viable state. That the proliferation-arrested TRPM7-deficient cells are viable is evidenced by their ability to both

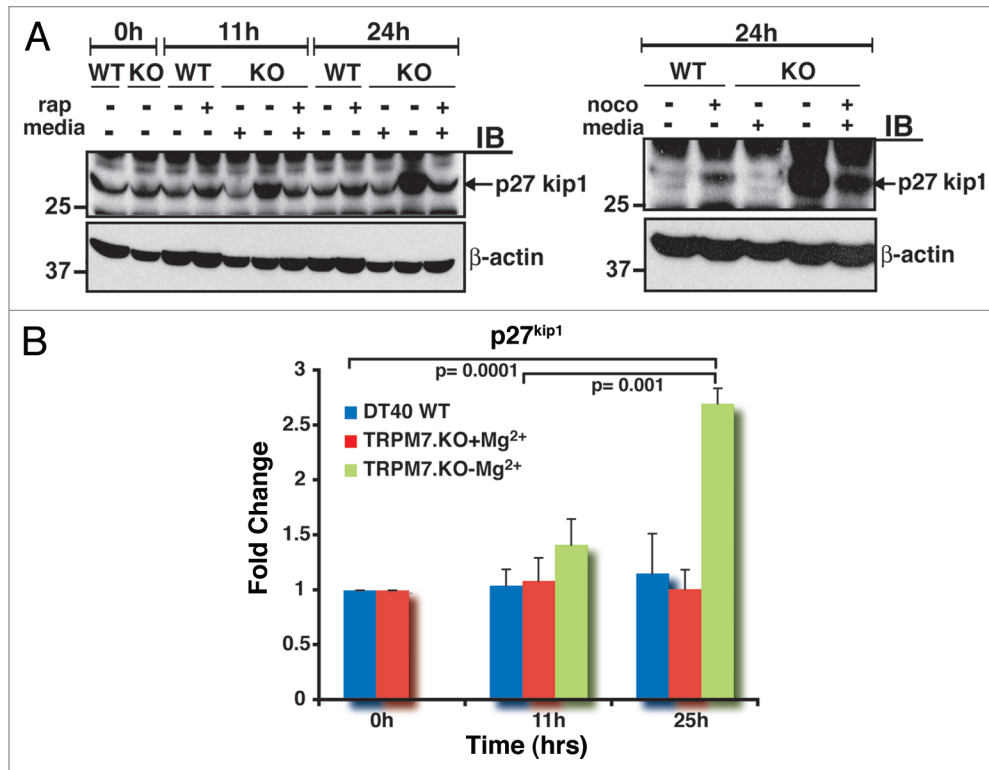


Figure 2. A signature protein of cellular quiescence, p27^{kip1}, is upregulated in proliferation-arrested TRPM7-KO cells. (A) Levels of p27^{kip1} are significantly elevated in non-proliferating TRPM7-KO cells. Left part: WT, TRPM7-KO cells and TRPM7-KO cells transitioned to regular media were analyzed for p27 expression at 11 and 24 hours post transition. At 11 hours, levels of p27 were elevated in TRPM7-deficient cells transitioned to regular media and were even significantly high at 24 hours post transition compared with WT cells growing in regular media and TRPM7-KO cells in growth-supporting media either with or without rapamycin, a mTOR inhibitor. Right part: Similar to the experiment in the left part, WT, proliferating TRPM7-deficient cells and non-proliferating TRPM7-KO cells were treated with a microtubule inhibitor, nocodazole for 24 hours. Exposure of cells to nocodazole resulted in p27 expression in both WT as well as proliferating TRPM7-KO cells but the levels were considerably lower than those observed in non-proliferating TRPM7-deficient cells. (B) Analysis of the fold change in p27^{kip1} from three independent experiments is shown. The graph shows averages plus standard errors of the means (error bars) and p values for the fold change were calculated using Student's t test. A 2.7-fold increase in p27 expression was observed by 25 hours in TRPM7-KO cells transitioned to regular media and found to be statistically significant compared to WT cells in regular media at 0 and 11 hours ($p = 0.0001$ and $p = 0.0006$) as well as TRPM7-KO cells cultured in growth-supporting media with 15 mM Mg²⁺ at similar time points ($p = 0.0001$ and $p = 0.001$).

maintain a constant level of CFSE staining, and their retention of normal membrane integrity as assessed by propidium iodide staining (Suppl. Fig. 2). Provision of 15 mM supplemental Mg²⁺ to TRPM7-KO cells after 24 hrs supported cell cycle re-entry as indicated by a progressive loss of CFSE fluorescence in the TRPM7-KO population, and sorting based on cell size demonstrated that cells with either 2n or 4n DNA content are able to resume cell division (Fig. 1B, (4n cells are distinctly larger than those which have arrested with 2n DNA content;⁷ Suppl. Fig. 1)). Taken together with our previous analyses of DNA content and cell size,¹ these results indicate that TRPM7-deficient cells placed in regular media have reversibly exited the cell cycle to a state of quiescence. The smaller cells with 2n DNA content would be predicted to have exited after mitosis, suggesting they have entered a state of G₀, while the larger cells arrested with 4n DNA content appear to have exited somewhere in G₂, as has been previously reported to occur in other eukaryotic systems.^{8,9}

p27^{kip1} is upregulated in quiescent TRPM7-deficient lymphocytes. To further define the properties of proliferation-arrested

TRPM7-deficient cells, we evaluated the expression level of a key cell cycle regulator-p27^{kip1}.¹⁰ The CDK inhibitor p27^{kip1} (hereafter p27/p27^{kip1}) is thought to provide a crucial block for the transition of cells from G₀ through G₁ into S phase in diverse type of cells, including lymphocytes (reviewed in ref. 11). A growing body of literature suggests that one of the important signatures of cellular quiescence is an elevation in p27 levels, since its abundance has been observed to precipitously decrease as quiescent primary lymphocytes progress out of G₀ into G₁ upon mitogen/growth-factor stimulation.^{12,13}

To evaluate the p27^{kip1} status of proliferation-arrested TRPM7-deficient cells, TRPM7-deficient cells were analyzed for p27^{kip1} expression at 11 and 24 hrs after transition to regular media with physiologic levels of Mg²⁺, and compared to both WT DT40 cells in regular media and TRPM7-KO cells in growth-supporting media with 15 mM Mg²⁺. Comparative analysis showed that p27 expression was significantly elevated in TRPM7-deficient cells growing in regular media by 11 hrs, followed by an even further augmentation at 24 hrs (Fig. 2A, left part). These levels

Figure 3 (See opposite page). Non-proliferating TRPM7-deficient cells display low RNA content, significantly downregulated store-operated Ca^{2+} entry (SOCE) and oxygen consumption rate analogous to primary lymphocytes. (A) TRPM7-deficient cells transitioned to regular media exhibit reduced RNA and SOCE. Top left part: Flow cytometric analysis of WT cells in regular media, TRPM7-KO cells in growth-supporting media with/without rapamycin (26 hrs) and TRPM7-KO cells transitioned to regular media for 26 hours stained with DAPI for DNA (Y-axis) and Pyronin Y for RNA (X-axis) content. TRPM7-deficient cells in regular media exhibited a high proportion of Pyronin Y negative cells that are in G_0 compared to proliferating WT and TRPM7-KO cells as well as TRPM7-KO treated with rapamycin. The analysis was performed on the same day. Top middle part: TRPM7-KO cells were transitioned to regular media for 24 and 48 hours and their surface IgM expression was analyzed with an anti-IgM antibody directly conjugated to FITC (Bethyl laboratories). WT cells in regular media and TRPM7-KO cells in Mg^{2+} -supplemented media displayed higher surface IgM expression compared to TRPM7-deficient cells transitioned to regular media, consistent with their smaller cell size. Top right part: Store-operated calcium entry was evaluated in WT, TRPM7-KO cells in growth-supporting media with 15 mM Mg^{2+} and TRPM7-KO cells induced into a non-proliferative state upon transition to regular media for 24 and 52 hours. Cells were labeled with Ca^{2+} -binding dye, indo-1 and acquired on BD LSRII cytometer. Flow kinetic profiles are shown comparing the mean indo-1 ratio (violet/blue) as a function of time before and after stimulation with M4 followed by addition of 1 mM Ca^{2+} . A significant reduction in SOCE was observed in TRPM7-deficient cells cultured in regular media for 24 and 52 hours. Lower left part: WT cells growing in regular media, TRPM7-KO cells in Mg^{2+} -supplemented media and proliferation-arrested TRPM7-deficient cells (24 hours) were analyzed for SOCE upon treatment with thapsigargin. Flow kinetic profiles obtained were similar to what was observed with M4 stimulation (top right part). Lower right part: Changes in intracellular calcium over time for TRPM7-KO cells in media with supplemental Mg^{2+} and in regular media without 15 mM Mg^{2+} . The response from the whole population of cells from one representative experiment is shown. Upon addition of 1 mM Ca^{2+} , the ion flux in the proliferation-arrested TRPM7-deficient cells (right dot plot) is significantly reduced as compared to the cells in growth-supporting media (left dot plot). Live/dead cell differentiation was done by propidium iodide (PI) staining in all experiments. (B) Proliferation-arrested TRPM7-deficient cells lack Crabtree effect despite functional mitochondria. Left part: A flow cell approach was used for measuring the oxygen consumption rate (OCR) in proliferation-arrested TRPM7-deficient cells compared to WT DT40 cells cultured in regular media and TRPM7-deficient cells in growth-supporting media. Non-proliferating TRPM7-KO cells showed a considerable drop in the OCR, closely resembling the oxygen consumption rate of quiescent primary lymphocytes, when compared to WT and TRPM7-KO cells in Mg^{2+} -supplemented media. Right part: Mitochondrial function was evaluated by determination of mitochondrial membrane potential in WT, TRPM7-deficient cells in growth-supporting media and non-proliferating TRPM7-KO cells transitioned to regular media for 24 hours. While cells labeled with MitoProbe JC-1 displayed mitochondria that retained their normal membrane potential, treatment with CCCP for 5 minutes led to a complete disruption of their mitochondrial electrochemical gradient, which was observed as the shift of JC-1 to its monomeric form (FL-1).

were prominently higher than those observed in TRPM7-KO and WT cells treated with rapamycin, an inhibitor of mTOR that induces G_1 arrest. Treatment of WT and TRPM7-KO cells with nocodazole, a microtubule inhibitor that induces G_1 and G_2 arrest,¹⁴ also resulted in expression of p27 at 24 hrs, but again not to the degree observed in TRPM7-KO cells cultured in regular media (Fig. 2A, right part). Quantitative fold change analysis showed that relative to proliferating WT cells in regular media and TRPM7-KO cells in growth-supporting media, TRPM7-deficient cells in regular media exhibited a 2.7-fold increase in p27 expression at 25 hrs, which was found to be statistically significant (Fig. 2B). In conjunction with previous observations on the time course of PI3K-Akt signaling downregulation that occurs in TRPM7-deficient cells,¹ these results suggest that accumulation of p27 follows the downregulation of PI3K-Akt growth signaling as TRPM7-deficient cells exit the cell cycle, a correlation also observed in primary lymphocytes transitioning from proliferation to quiescence (reviewed in ref. 15, 16). Taken together, these data indicate that proliferation-arrested TRPM7-deficient lymphocytes exhibit a key molecular signature of cell cycle exit to quiescence—accumulation of high levels of p27^{Kip1}.

Reduced RNA, store-operated calcium entry (SOCE) and altered energy processes characterize quiescent TRPM7-deficient cells. A defining characteristic of quiescent cells is the presence of extremely low levels of RNA compared to cycling cells in G_1 , S or G_2 -M phase. Cellular RNA levels can be defined in relation to their cell cycle position by sequential staining of cells with DAPI (binds to DNA), followed by Pyronin Y (PY) staining (binds to RNA only, after DNA binding sites are saturated with DAPI).¹⁷ In order to assess RNA levels and cell cycle status of TRPM7-KO lymphocytes transitioned to regular media for 26 hrs, we compared them with sequentially labeled WT cells

in regular media, TRPM7-KO cells in growth-supporting media and TRPM7-KO cells in growth-supporting media treated with rapamycin for 26 hrs. We observed a significant proportion of PY^{low} cells in TRPM7-deficient cells in regular media (79%), suggesting that the majority of cycling cells had become quiescent (Fig. 3A, top left part; reviewed in ref. 18).

To further define the nature of the quiescent state that TRPM7-deficient cells had entered, we examined the magnitude of store-operated calcium entry in TRPM7-KO cells transitioned to regular media, which has been shown to be diminished in quiescent lymphocytes,¹⁹ and cellular energy utilization, which is also reduced and dominated by aerobic/mitochondrial ATP production in quiescence (reviewed in ref. 20).

Engagement of antigen receptors on lymphocytes is followed by calcium-release from endoplasmic reticulum (ER) stores into the cytosol, resulting in the activation of store-operated calcium entry via calcium-release-activated calcium (CRAC) channels on the plasma membrane (reviewed in ref. 21, 22). The magnitude of store-operated Ca^{2+} entry in primary lymphocytes driven to actively proliferate has recently been shown to be markedly enhanced relative to those that are quiescent.¹⁹ To evaluate store-operated calcium entry in proliferation-arrested TRPM7-deficient lymphocytes, we compared WT DT40 cells in regular media with TRPM7-deficient cells maintained either in Mg^{2+} -supplemented media or those that had been transitioned to regular media for 24 hours to induce a state of non-proliferation. Each cell type was activated with M4, an IgM monoclonal antibody that activates the BCR or thapsigargin, an inhibitor of the SERCA (sarco endoplasmic reticulum Ca^{2+} ATPase) pump responsible for Ca^{2+} transport into the ER.^{23,24} Surface expression of IgM was confirmed with a specific anti-IgM antibody (Fig. 3A, top middle part) and release of calcium from intracellular stores

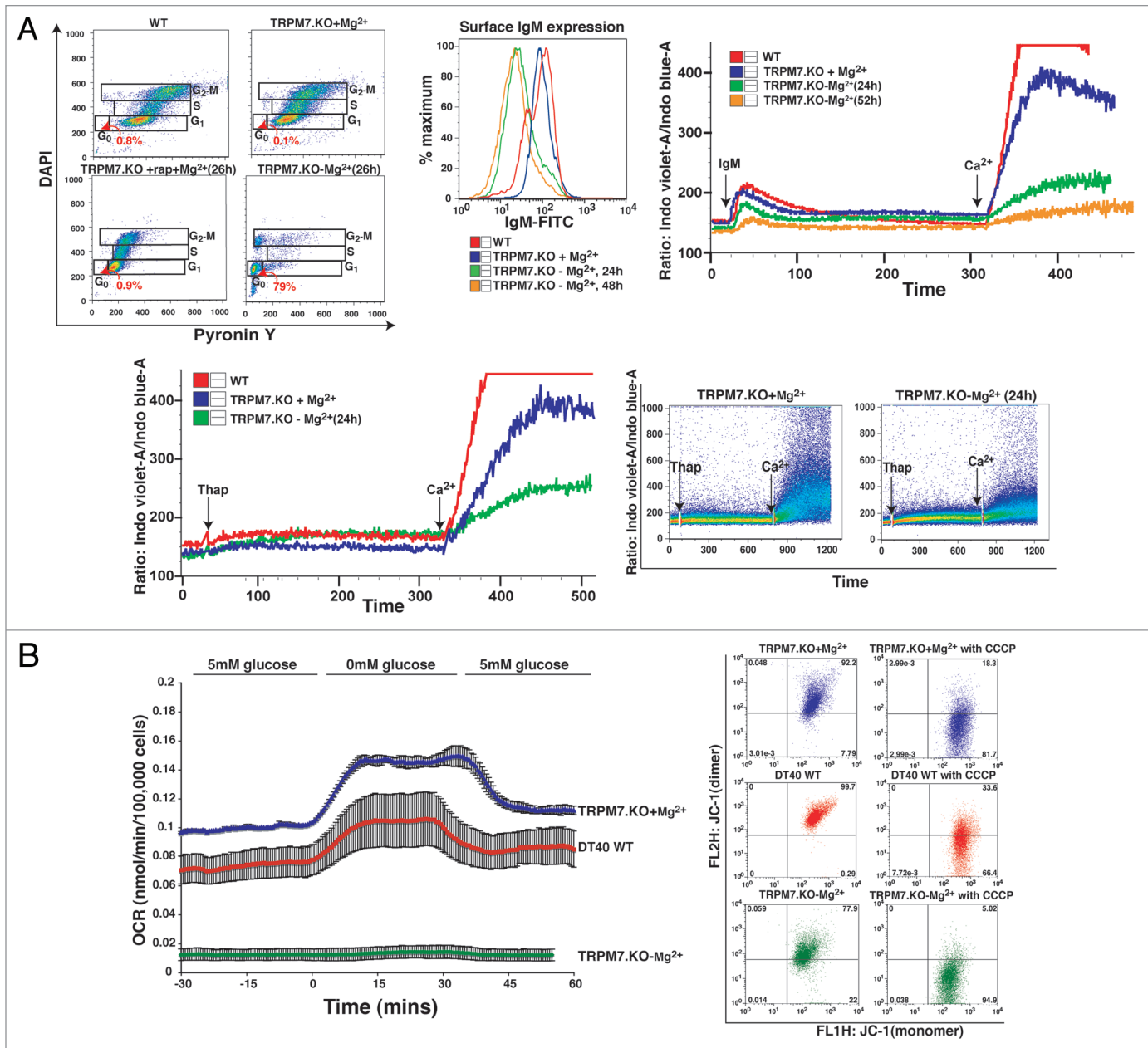


Figure 3. For figure legend, see page 3568.

followed by entry of extracellular Ca^{2+} was monitored using the Ca^{2+} -binding dye, indo-1.²⁵ We observed a considerable reduction in anti-IgM evoked intracellular Ca^{2+} depletion from ER stores and extracellular Ca^{2+} entry in TRPM7-deficient B-lymphocytes transitioned to regular media for 24 hrs, as compared with WT cells growing in regular media and TRPM7-KO cells cultured in growth-supporting media (Fig. 3A, top right part). Similar results were obtained upon depleting the intracellular Ca^{2+} stores with thapsigargin. Together, these results demonstrate that proliferation-arrested TRPM7-deficient cells exhibit downregulated store-operated Ca^{2+} entry, which is both qualitatively and quantitatively similar to that observed in quiescent primary lymphocytes (Fig. 3A, lower part and reviewed in ref. 19).

An additional fundamental property of cellular quiescence is the nature of cellular energy metabolism. In lymphocytes, energy metabolism in quiescence is dominated by respiration, and thus quiescent lymphocytes exhibit low rates of glucose utilization and oxygen consumption (reviewed in ref. 26, 27). In contrast, rapidly proliferating lymphocytes exhibit a high rate of energy utilization that is associated with a shift in carbon flux through glycolysis into metabolic pathways, which generate biosynthetic building blocks required to add cell mass. The shift of carbon flux into glycolytic metabolism results in a significant fraction of energy production via glycolysis in addition to a high rate of ongoing respiration, and is thus known as aerobic glycolysis. A sinequa-non of ongoing aerobic glycolysis is a paradoxical increase in

Figure 4 (See opposite page). Induction of TRPM7 by doxycycline or provision of supplemental Mg^{2+} allows proliferation-arrested TRPM7-deficient cells to re-enter the cell cycle. (A) TRPM7- Δ -kinase cells transitioned to regular media display proliferation arrest in both G_0/G_1 and G_2 populations. TRPM7- Δ -kinase cells cultured in growth-supporting media were transitioned to regular media for 24 hours following which they were sorted on the basis of their size scatter (as shown in B) into G_0/G_1 and G_2 populations, commensurate to TRPM7-KO cells. Cells were labeled with CFSE along with positive controls-proliferating TRPM7- Δ -kinase cells in growth supporting media and TRPM7- Δ -kinase cells induced with doxycycline. Comparative analysis showed that while the positive controls proliferated and led to subsequent partitioning of the dye, proliferation arrested and sorted cells in G_0/G_1 and G_2 transitioned to regular media, displayed stable CFSE staining and did not undergo any further cell divisions. (B) Proliferation-arrested TRPM7- Δ -kinase cells are able to recommence proliferation upon doxycycline induction or provision of 15 mM Mg^{2+} . CFSE-labeled sorted G_0/G_1 and G_2 populations were either induced with doxycycline (dox; 1 μ g/ml) for TRPM7- Δ -kinase expression or replenished with supplemental Mg^{2+} . By 72 hours, a sizable magnitude of cells were able to exit cell cycle arrest in both G_0/G_1 as well as G_2 populations and resume cell division. However, TRPM7- Δ -kinase cells provided with doxycycline exhibited a lag in resumption of cell division as compared to the cells provided with supplemental Mg^{2+} , which could be attributed to the time required by the dox-induced cells to initiate protein synthesis. (C) TRPM7- Δ -kinase expression drives both G_0/G_1 and G_2 proliferation-arrested cells to reenter cell cycle. TRPM7- Δ -kinase cells transitioned to regular media for 24 hours were sorted into G_0/G_1 and G_2 populations and labeled with CFSE. Left part: Post-sort, cells were transitioned to growth-supporting media or induced with doxycycline. Non-proliferating, stable CFSE labeled cells and proliferating, CFSE-low populations from doxycycline-induced G_2 cells were further sorted at 64 hours, lysed and immunoprecipitated with anti-HA antibody. The immunoprecipitates were run on 8% SDS-PAGE and immunoblotted with anti-HA antibody for expression analysis of HA-tagged TRPM7- Δ -kinase. Right part: Similar CFSE-labeled populations, as mentioned in the left part, were sorted at 87 hours for doxycycline induced G_0/G_1 cells (stable CFSE labeled and CFSE low populations) expressing TRPM7- Δ -kinase and uninduced cells provided with 15 mM supplemental Mg^{2+} . Cells were fixed, permeabilized and labeled with anti-HA/anti-mouse PE antibodies for detection of TRPM7- Δ -kinase by flow cytometry on BD LSRII. A dramatic increase in fluorescence was observed in proliferating CFSE-low, doxycycline-induced G_0/G_1 population as compared to the non-proliferating, stable CFSE-labeled cells. A small shift in fluorescence was also observed in the uninduced CFSE-low G_0/G_1 cells, which could be attributed to a leaky promoter.

oxygen consumption upon glucose restriction, as restriction of cellular energy production via glycolysis is made up for via a shift of energy metabolism towards respiration, a phenomenon known as the Crabtree effect.²⁸ Thus, quiescent cells exhibit a low rate of oxygen consumption and lack a “Crabtree effect” upon glucose restriction.²⁹

To evaluate the metabolism of proliferation-arrested TRPM7-deficient cells, we used a flow cell with continuous on line monitoring of oxygen consumption through detection of the phosphorescence lifetime of an oxygen-sensitive dye (platinum tetrapentafluorophenyl porphyrin).³⁰ Using this approach, the oxygen consumption rate (OCR) was measured as environmental glucose was varied from 0 to 5 mM glucose (Fig. 3B, left part). Oxygen consumption rates of proliferating WT cells in regular media and TRPM7-deficient cells in growth-supporting media were approximately 0.7 nmol/10⁵ cell/minute, and markedly increased upon transition to glucose deprivation conditions (Fig. 3B, left part), consistent with their cell metabolism existing in a state of aerobic glycolysis required to support continuous rapid proliferation. In contrast, the OCR of proliferation-arrested TRPM7-KO cells decreased to an absolute rate approaching what has previously been reported for quiescent primary thymocytes,^{26,31} and essentially no change in OCR was observed upon glucose deprivation. These observations indicate that the proliferation-arrested TRPM7-deficient cells have transitioned to a metabolic state in which they have low energy needs, and those needs are met primarily through mitochondrial respiration.²⁰ Mitochondrial function was also directly evaluated in the proliferation-arrested TRPM7-deficient cells by assessment of the mitochondrial membrane potential using MitoProbe JC-1, which reports high mitochondrial membrane potential as fluorescent J-aggregates (red/FL-2), and loss of membrane potential as a shift of the dye to its monomeric form (green/FL-1).³² Significant mitochondrial membrane potentials were apparent in WT DT40 cells cultured in regular media, TRPM7-deficient cells maintained in Mg^{2+} supplemented media and proliferation-arrested

TRPM7-deficient cells maintained in regular media (Fig. 3B, right part), as can be seen through the sizeable collapse of the membrane potential (and shift to green/FL-1 fluorescence) resulting from treatment with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) in each type of cell (Fig. 3B, right part).

Proliferation-arrested, quiescent TRPM7-deficient lymphocytes can resume cell cycle. The cumulative results above indicate that proliferation-arrested TRPM7-deficient cells acquire every major reported quiescence-associated molecular and physiological signature. To determine whether TRPM7 channel function is sufficient to reverse an established quiescent state, we made use of a previously described TRPM7-deficient cell line which expresses a TRPM7 kinase domain deletion mutant under the control of a doxycycline-regulated promoter (TRPM7- Δ -kinase, reviewed in ref. 3). This cell line was chosen over TRPM7-KO cells complemented with full-length TRPM7 (cWT), as we have previously shown that cWT cells continue to cycle even when uninduced, apparently due to low basal expression level of the transfected protein driven by a leaky promoter.³ In contrast, when cultured in standard tissue culture media in the absence of doxycycline, TRPM7- Δ -kinase overexpressing cells undergo proliferative arrest indistinguishable from that of uncomplemented TRPM7-deficient cells; when doxycycline is added to the tissue culture media, these cells regain the ability to proliferate in standard tissue culture media, albeit at a reduced rate relative to growth in Mg^{2+} supplemented media.³ Analogous to earlier experiments with TRPM7-deficient cells, untreated TRPM7- Δ -kinase cells cultured in media with supplemental Mg^{2+} were transitioned to regular media for 24 hours, resulting in their proliferative arrest. Arrested cells were sorted on the basis of their forward scatter into small (putative 2n/ G_0) and large (putative 4n/ G_2 -quiescent) populations, labeled with CFSE and were then either kept in regular media, transitioned back to growth-supporting media with supplemental Mg^{2+} or TRPM7- Δ -kinase expression was induced with doxycycline. Both the sorted putative G_0 and

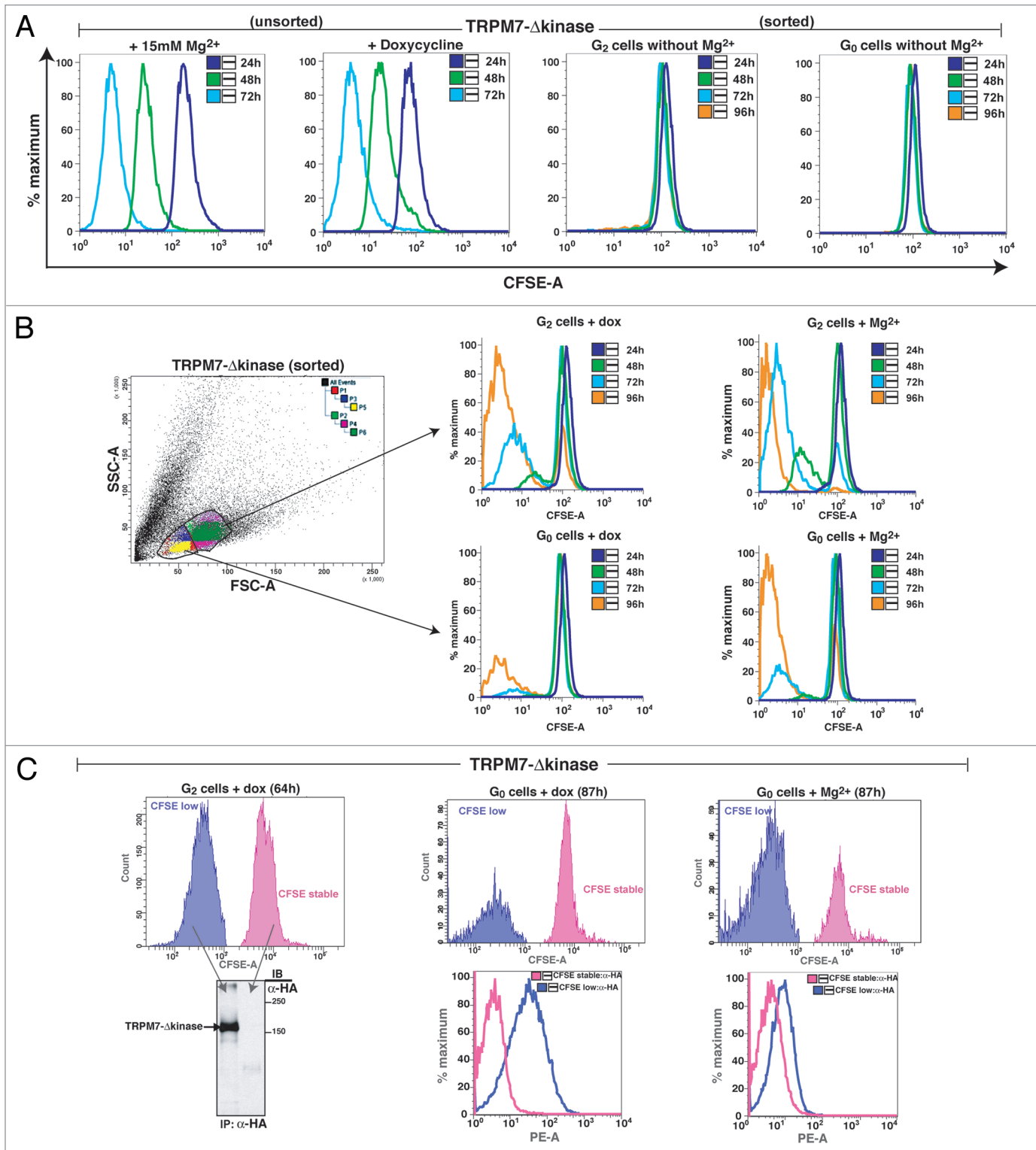


Figure 4. For figure legend, see page 3570.

G₂-quiescent populations kept in standard media displayed stable CFSE staining identical to that observed in proliferation-arrested TRPM7-KO cells (Fig. 4A). Upon provision of 15 mM Mg²⁺, a proportion of cells in both putative G₀ and G₂-quiescent populations were able to re-enter the cell cycle (Fig. 4B). This result is

identical to that observed for TRPM7-KO cells, and confirms that Mg²⁺ uptake is necessary and sufficient for resumption of proliferation from a state of quiescence for TRPM7- Δ kinase cells. Induction of TRPM7- Δ kinase expression via doxycycline was also able to support a proportion of cells in both G₀/

G₁ and G₂ populations to re-enter the cell cycle. To determine whether TRPM7-Δ-kinase expression correlated with cell cycle re-entry, we analyzed TRPM7-Δ-kinase expression in uninduced and induced cells maintained in the various conditions (Fig. 4C). Consistent with TRPM7-dependent Mg²⁺ uptake playing an essential role in supporting cell cycle re-entry of quiescent cells, the induced cells re-entering the cell cycle show substantial expression of TRPM7-Δ-kinase, while those unable to re-enter the cell cycle lack TRPM7-Δ-kinase expression (Fig. 4C).

Discussion

Overall, our data suggests that culture of TRPM7-deficient cells in standard tissue culture media results in their entering a state of quiescence: they reversibly cease proliferation, upregulate expression of p27^{kip1}, display low RNA content, downregulate store-operated Ca²⁺ entry, and adopt a metabolic state dominated by mitochondrial respiration. As either supplemental Mg²⁺ or induction of TRPM7 expression are able to initiate cell cycle re-entry after proliferation arrest, uptake of extracellular Mg²⁺ via TRPM7 is identified as an essential biochemical event regulating transitions from quiescent to proliferative metabolism in lymphocytes. While the conditions used to induce quiescence also result in a significant fraction of TRPM7-deficient cells irreversibly exiting the cell cycle, this is attributable to those cells experiencing a prolonged period of quiescence, which is frequently observed to lead to irreversible cell cycle exit and senescence.^{33,34}

An intriguing question raised by our results is the nature of the role TRPM7-dependent Mg²⁺ uptake has in supporting the transition from quiescent to proliferative physiology. Previous models have posited that glucose uptake is a key event driving the transition to aerobic glycolysis upon lymphocyte activation (reviewed in ref. 35). Because both respiration and aerobic glycolysis utilize glucose and other fuel substrates, it is difficult to see how uptake of fuel substrates alone might lead to such a marked metabolic alteration. However, the respective requirements of respiration and aerobic glycolysis for external Mg²⁺ uptake are entirely divergent: respiration recycles MgADP to MgATP, and thus does not affect the size of the cellular adenine nucleotide pool. In contrast, aerobic glycolysis is associated with massive de novo expansion of the adenine nucleotide pool, resulting in generation of new molecules of ADP, each of which requires a new Mg²⁺ ion to be taken up from the extracellular milieu. Thus, enhanced Mg²⁺ uptake is only required when a cell must expand its adenine nucleotide pool—precisely the situation which arises when a cell is activated to rapidly proliferate and must adjust its metabolism accordingly. If TRPM7-dependent Mg²⁺ uptake is generally required to initiate metabolic transitions associated with rapid proliferation, such as those that occur during malignant transformation, then targeting of Mg²⁺ uptake via TRPM7 could provide a novel strategy for inhibition of the growth of diverse types of malignant cells.

Materials and Methods

Cell culture. DT40 B-lymphocytes were maintained in Roswell Park Memorial Institute (RPMI 1640) (Mediatech) with 10%

fetal bovine serum (FBS), 1% chicken serum, 10 U/ml penicillin/streptomycin, 2 mM glutamine and 50 μg/ml blasticidin. For cell division experiments, cells were stained with CFSE according to the manufacturer's instructions (Invitrogen). CFSE-labeled cells were cultured in complete RPMI with 10% FBS, 1% chicken serum, 10 U/ml penicillin/streptomycin and 2 mM glutamine. In some experiments supplemental Mg²⁺, rapamycin, nocodazole and staurosporine were added at final concentrations of 15 mM, 100 nM, 3.3 μM and 1 μM, respectively.

Measurement of intracellular Ca²⁺ and Ca²⁺ flux. For measurement of [Ca²⁺]_i, cells were washed and resuspended in PBS containing 5% FBS at 10⁶–10⁷ cells/ml. Cell permeant indo-1-acetoxymethyl (Invitrogen) was added at a final concentration of 7 μM, and incubation was performed for 30 min at 37°C. The indo-1 fluorescence ratio (indo-1 was excited at 325 nm and emission detected using a 530/30 nm filter for Ca²⁺ free and a 405/20 nm filter for Ca²⁺ bound) of the cells was acquired as a function of time using a flow cytometer (BD-LSR II; Becton Dickinson). For each experiment, collection of 1-minute baseline measurement was followed by an initial stimulation with either 10 μg/ml anti-chicken IgM (Bethyl laboratories) or 1 μM thapsigargin (Biomol) with subsequent addition of 1 mM Ca²⁺ after 10–20 minutes, as indicated. For the purposes of direct comparison, the data presented in the figures are from single experiments performed in parallel though experiments were repeated two to three times with similar results. The kinetics of the data was analyzed with FlowJo software (TreeStar).

Immunoprecipitation, electrophoresis and western blotting. Untreated or treated cells were washed once with PBS and whole cell lysates were prepared by lysing the cells in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA] and complete mini-protease-inhibitor cocktail without EDTA (Roche), used according to manufacturer's instructions. The lysates were rotated for 45 min at 4°C and cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration of lysates was determined by Bicinchoninic acid (BCA; Pierce) assay using the manufacturer's specifications and anti-HA antibody (Cell Signaling) was used for immunoprecipitation of HA-tagged TRPM7-Δ-kinase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli. Aliquots of supernatants/immunoprecipitates were separated on SDS-PAGE gels (10–12% for p27 and 6% for TRPM7-Δ-kinase) and analyzed by immunoblotting (IB). The proteins were transferred to 0.45 μm pore size polyvinylidene fluoride membranes (PVDF; Millipore) in transfer buffer (39 mM glycine, 48 mM tris base and 20% methanol) for 1–2 hours at 4°C. Membranes were blocked in 5% blocking buffer (5% w/v nonfat dry milk in TBS-0.1% Tween-20) for 1 hr at room temperature. Primary-antibody incubations were done overnight at the dilutions specified by the vendor. Incubations with the secondary antibody were performed with peroxidase-coupled anti-rabbit/anti-mouse immunoglobulin in 5% blocking buffer and the bound antibody was detected by ECL Chemiluminescence Detection System (Amersham).

Measurement of oxygen consumption rate (OCR). OCR was measured in a perfusion system that allows for continuous

measurement and flow rate was set to 80 microl/min; chamber volume was 400 microl. Cells (5–20 million) were loaded into the chamber with Cytodex beads (Amersham Biosciences) and sandwiched between two layers of Cytopore beads (Amersham Biosciences). OCR was calculated as the flow rate times the difference between inflow and outflow oxygen tension, which was measured by detecting the phosphorescence lifetime of an oxygen-sensitive dye that was painted on the inside of the perfusion chamber. Phosphorescent lifetimes were monitored using an MFPPF-100 multifrequency phase fluorometer lifetime measurement system made by TauTheta Instruments (Boulder, CO) where the end of the excitation light guide (2 mm fiber optic patch cord; TauTheta part no. SFO-026) was illuminated by a 405 nm light-emitting diode that was just touching the outside of the glass opposite where the dye was painted and the detecting light guide was positioned at a 90° angle.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured by MitoProbe™ JC-1 Assay Kit for Flow Cytometry (M34152; Invitrogen). Briefly, cells were suspended in RPMI at approximately 1×10^6 cells/ml and 10 μ l of 200 μ M JC-1 was added. Cells were incubated at 37°C, 5% CO₂ for 20 minutes and for the control sample, 1 μ l of 50 mM mitochondrial membrane potential disruptor, CCCP (carbonyl cyanide 3-chlorophenylhydrazine) was added and cells were further incubated at 37°C for 5 minutes. After a wash with PBS, cells were resuspended in 500 μ l of PBS by gentle flicking of the tube and analyzed by flow cytometry on BD FACSCalibur.

References

- Sahni J, Scharenberg AM. TRPM7 ion channels are required for sustained phosphoinositide 3-kinase signaling in lymphocytes. *Cell Metab* 2008; 8:84-93.
- Sahni J, Nelson B, Scharenberg AM. SLC41A2 encodes a plasma-membrane Mg²⁺ transporter. *Biochem J* 2007; 401:505-13.
- Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, et al. Regulation of vertebrate cellular Mg²⁺ homeostasis by TRPM7. *Cell* 2003; 114:191-200.
- Yusuf I, Fruman DA. Regulation of quiescence in lymphocytes. *Trends Immunol* 2003; 24:380-6.
- Giaretti W, Abmayr W, Dormer P, Santi L. The G₀ in equilibrium G₁ transitions of human lymphocytes as monitored by quantitative 14C-uridine autoradiography and high-resolution image analysis. *Cytometry* 1985; 6:219-25.
- Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 2000; 243:147-54.
- Gerson DF, Kiefer H. Intracellular pH and the cell cycle of mitogen-stimulated murine lymphocytes. *J Cell Physiol* 1983; 114:132-6.
- Negre N, Ghysen A, Martinez AM. Mitotic G₂-arrest is required for neural cell fate determination in *Drosophila*. *Mech Dev* 2003; 120:253-65.
- Wei W, Nurse P, Broek D. Yeast cells can enter a quiescent state through G₁, S, G₂, or M phase of the cell cycle. *Cancer Res* 1993; 53:1867-70.
- Kaldis P. Another piece of the p27^{Kip1} puzzle. *Cell* 2007; 128:241-4.
- Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer* 2008; 8:253-67.
- Coats S, Flanagan WM, Nourse J, Roberts JM. Requirement of p27^{Kip1} for restriction point control of the fibroblast cell cycle. *Science* 1996; 272:877-80.
- Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell* 2008; 14:159-69.
- Blajeski AL, Phan VA, Kottke TJ, Kaufmann SH. G(1) and G(2) cell cycle arrest following microtubule depolymerization in human breast cancer cells. *J Clin Invest* 2002; 110:91-9.
- Kops GJ, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffey PJ, et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 2002; 22:2025-36.
- Tzachanis D, Lafuente EM, Li L, Boussiotis VA. Intrinsic and extrinsic regulation of T lymphocyte quiescence. *Leuk Lymphoma* 2004; 45:1959-67.
- Deng X, Ewton DZ, Friedman E. Mirk/Dyrk1B maintains the viability of quiescent pancreatic cancer cells by reducing levels of reactive oxygen species. *Cancer Res* 2009; 69:3317-24.
- Darzynkiewicz Z, Traganos F, Melamed MR. New cell cycle compartments identified by multiparameter flow cytometry. *Cytometry* 1980; 1:98-108.
- Lioudyno MI, Kozak JA, Penna A, Safrina O, Zhang SL, Sen D, et al. Orai1 and STIM1 move to the immunological synapse and are upregulated during T cell activation. *Proc Natl Acad Sci USA* 2008; 105:2011-6.
- Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* 2005; 5:844-52.
- Lewis RS. The molecular choreography of a store-operated calcium channel. *Nature* 2007; 446:284-7.
- Cahalan MD, Zhang SL, Yeromin AV, Ohlsen K, Roos J, Stauderman KA. Molecular basis of the CRAC channel. *Cell Calcium* 2007; 42:133-44.
- Lievremont JP, Bird GS, Putney JW Jr. Mechanism of inhibition of TRPC cation channels by 2-aminoethoxy-diphenylborane. *Mol Pharmacol* 2005; 68:758-62.
- Pogue SL, Kurosaki T, Bolen J, Herbst R. B cell antigen receptor-induced activation of Akt promotes B cell survival and is dependent on Syk kinase. *J Immunol* 2000; 165:1300-6.
- June CH, Rabinovitch PS. Flow cytometric measurement of intracellular ionized calcium in single cells with indo-1 and fluo-3. *Methods Cell Biol* 1990; 33:37-58.
- Buttgereit F, Burmester GR, Brand MD. Therapeutically targeting lymphocyte energy metabolism by high-dose glucocorticoids. *Biochem Pharmacol* 2000; 59:597-603.
- Kraus S, Brand MD, Buttgereit F. Signaling takes a breath—new quantitative perspectives on bioenergetics and signal transduction. *Immunity* 2001; 15:497-502.
- Ibsen KH. The Crabtree effect: a review. *Cancer Res* 1961; 21:829-41.
- Guppy M, Greiner E, Brand K. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *Eur J Biochem* 1993; 212:95-9.
- Gilbert M, Jung SR, Reed BJ, Sweet IR. Islet oxygen consumption and insulin secretion tightly coupled to calcium derived from L-type calcium channels but not from the endoplasmic reticulum. *J Biol Chem* 2008; 283:24334-42.
- Herst PM, Berridge MV. Cell surface oxygen consumption: a major contributor to cellular oxygen consumption in glycolytic cancer cell lines. *Biochim Biophys Acta* 2007; 1767:170-7.
- Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci USA* 1991; 88:3671-5.

Acknowledgements

We would like to thank Dr. Sarah Andrews and Mr. Ashok Bandaranayake (University of Washington and Seattle Children's Research Institute, Seattle, WA) for their helpful suggestions. This work was supported in part by NIH grants to A.M.S. and I.R.S. [DK17047 (the DERC Islet Core)]. We gratefully acknowledge the expert assistance of the Diabetes and Obesity Center of Excellence, University of Washington.

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/SahniCC9-17-sup.pdf

-
33. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007; 8:729-40.
 34. Toledo LI, Murga M, Gutierrez-Martinez P, Soria R, Fernandez-Capetillo O. ATR signaling can drive cells into senescence in the absence of DNA breaks. *Genes Dev* 2008; 22:297-302.
 35. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. *J Leukoc Biol* 2008; 84:949-57.