Original Research

Redox and mTOR-dependent regulation of plasma lamellar calcium influx controls the senescence-associated secretory phenotype

Akshaya Chandrasekaran[1], May Y Lee[1], Xuexin Zhang[2], Shaheen Hasan[1], Habben Desta[1], Scott A Tenenbaum[1] and J Andrés Melendez[1]

[1]SUNY Polytechnic Institute, Colleges of Nanoscale Science and Engineering, Albany, NY 12203, USA; [2]College of Medicine, Penn State University, Hershey, PA 17033, USA

Corresponding author: J Andrés Melendez. Email: jmelendez@sunypoly.edu

Impact statement

Through its ability to evoke responses from cells in a paracrine fashion, the senescence-associated secretory phenotype (SASP) has been linked to numerous age-associated disease pathologies including tumor invasion, cardiovascular dysfunction, neuroinflammation, osteoarthritis, and renal disease. Strategies which limit the amplitude and duration of SASP serve to delay age-related degenerative decline. Here we demonstrate that the SASP regulation is linked to shifts in intracellular Ca^{2+} homeostasis and strategies which rescue redox-dependent calcium entry including enzymatic H_2O_2 scavenging, TRP modulation, or mTOR inhibition block SASP and TRPC6 gene expression. As Ca^{2+} is indispensable for secretion from both secretory and non-secretory cells, it is exciting to speculate that the expression of plasma lamellar TRP channels critical for the maintenance of intracellular Ca^{2+} homeostasis may be coordinately regulated with the SASP.

Abstract

Cellular senescence has evolved as a protective mechanism to arrest growth of cells with oncogenic potential but is accompanied by the often pathologically deleterious senescence-associated secretory phenotype (SASP). Here we demonstrate an H_2O_2-dependent functional disruption controlling senescence-associated Ca^{2+} homeostasis and the SASP. Senescent cells fail to respond to H_2O_2-dependent plasma lamellar Ca^{2+} entry when compared to pre-senescent cells. Limiting exposure to senescence-associated H_2O_2 restores H_2O_2-dependent Ca^{2+} entry as well as transient receptor potential cation channel subfamily C member 6 (TRPC6) function. SA-TRPC6 and SASP expression is blocked by restoring Ca^{2+} entry with the TRP channel antagonist SKF-96365 or by the mTOR inhibitors rapamycin and Ku0063794. Together, our findings provide compelling evidence that redox and mTOR-mediated regulation of Ca^{2+} entry through TRPC6 modulates SASP gene expression and approaches which preserve normal Ca^{2+} homeostasis may prove useful in disrupting SASP activity.

Keywords: Senescence, SASP, calcium, TRPC6, mTOR, hydrogen peroxide

***Experimental Biology and Medicine* 2020; 245: 1560–1570. DOI: 10.1177/1535370220943122**

Introduction

Cellular senescence was first described by Hayflick and Moorhead[1] in 1961, showing human cells in culture have a finite proliferative capacity, which describe the feature of "senescence" at the cellular level. Cellular senescence is a process of irreversible cell cycle arrest that can be induced by multiple factors, including telomere shortening, oxidative stress, and oncogene activation.[2–7] Cellular senescence, a tumor constraining mechanism characterized by irreversible growth arrest, participates in embryonic development, tissue repair, and wound healing.[8–12] Cellular senescence is implicated as causal in many age-associated pathologies as a result of the often detrimental senescence-associated secretory phenotype (SASP). The SASP involves secretion of soluble factors (interleukins, chemokines, and growth factors), degradative matrix metalloproteases (MMPs),

and insoluble extracellular matrix (ECM) components which alter the tissue microenvironment affecting cell behavior in a paracrine fashion.[13] The free radical theory of aging posits that accumulation of macromolecular damage occurs after a lifetime of exposure to oxidants.[4] Though often refuted, several tenets of this theory stand true including the two critical traits focusing on species-specific low mitochondrial ROS generation rates at complex I of the electron transport chain (ETC) and lowered levels of fatty acid unsaturation on cellular and mitochondrial membranes in long-lived animals.[14] The mechanistic control of senescence and SASP factors by reactive oxygen species (ROS) has also garnered significant focus.[15]

Calcium (Ca^{2+}) is a ubiquitous intracellular messenger that regulates an extensive repertoire of cellular processes, ranging from contraction, secretion, fertilization, and development, to the control of transcription, proliferation, as well as learning and memory.[16] The Ca^{2+} concentration in cells is sustained by concurrently acting "on" and "off" mechanisms that serve to raise or lower cytosolic Ca^{2+} levels. Transient receptor potential (TRP) channels comprise a functionally diverse family of 28 channel proteins that mediate the transmembrane flux of cations down their electrochemical gradients, resulting in increased intracellular Ca^{2+} and Na^{+} concentrations and cellular depolarization, with cation selectivity depending on pore structure and channel regulation. TRP channels act as cellular biosensors for environmental stimuli and are also responsive to changes in temperature and conditions inside the cell. Certain TRP channels are also being increasingly incriminated as crucial players in detecting cellular redox status with many channels emerging as targets of ROS-mediated regulation either directly through oxidative amino acid modifications or indirectly through second messengers.[17] The "calcium overload" hypothesis in aging is supported by several studies that have demonstrated that intracellular Ca^{2+} levels are typically elevated in aged cells as compared to young cells due to oxidative stress, inefficient Ca^{2+} handling systems, and loss of Ca^{2+} signaling homeostasis.[18] The link between calcium signaling and senescence has been extensively reviewed.[19] Senescence-inducing stresses, including telomere shortening, oncogene activation, and oxidative stress, can trigger activation of phospholipase C (PLC), which cleave PIP_2 into DAG and IP_3. IP_3 then binds to IP_3-gated calcium channel, which releases calcium from endoplasmic reticulum (ER), resulting rapid elevation of cytosolic calcium concentration.[20–23] In addition to IP_3 channel, silencing TRPM7 and TRPM8 can induce replicative senescence in pancreatic adenocarcinoma cells suggesting that these channels may be important for preventing oncogene-induced senescence, and promoting pancreatic tumorigenesis.[24,25] The Ca^{2+} overload can also enhance the deleterious effects of SASP via the activation of calpain and the subsequent processing and functionality of interleukin-1 alpha (IL-1α).[26]

The mammalian target of rapamycin (mTOR) complexes mTORC1 and mTORC2 play essential roles in regulating growth regulation, nutrient signaling, cytoskeletal organization, cell survival, and aging. mTOR signals also modulate senescence.[27] As mitochondria are the primary source of ROS production, it is not surprising that majority of oxidative stress induced senescent-associated changes are mitochondria dependent.[28] Mitochondria depleted cells display reduced senescence-associated (SA)-β-gal activity, formation of SA-heterochromatin foci, expression of p16 and p21, and ROS generation.[29] Mitochondrial dysfunction can lead to increased mitochondrial ROS, which trigger mTORC1 pathway, resulting in p53-mediated senescence activation.[30] In addition, current insight suggests that mTOR modulates SASP by regulating the translation of IL-1α and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2). IL-1α promotes NF-κB in a cell-autonomous manner to facilitate the transcription of SASP genes. Additionally, MAPKAPK2 phosphorylates and inhibits the RNA-binding protein ZFP36L1, thereby stabilizing SASP gene targets containing AREs (AU-rich elements).[31] Shifts in intracellular Ca^{2+} also play a part in mTOR signaling;[32] conversely, mTOR contributes to age-associated shifts in Ca^{2+} mobilization.[33]

Our findings illustrate that dysregulated Ca^{2+} signaling during senescence is affected by desensitization of redox-dependent Ca^{2+} channels. We found that enzymatic H_2O_2 removal, treatment with pharmacological Ca^{2+}-modulating agents, or mTOR inhibition can rescue defects in senescence-associated (SA) Ca^{2+} mobilization and limit SASP. Overall, our studies provide insights into the contributions of Ca^{2+} in senescence regulation as well as the potential to identify new therapeutic targets limiting SASP and potentially age-associated disease progression.

Materials and methods

Cell culture

Primary human fetal lung IMR-90 fibroblasts were cultured in MEM (Corning Cellgro) with 10% FBS (Biowest) and incubated at 21% or 3% oxygen tension at 37°C with 5% CO_2. The cells were lifted in 0.25% Trypsin-2.21 mM EDTA 1X (Corning) and serially passaged 1:4 for pre-senescent passages less than 20 and at 1:2 for passages beyond that. Cells <p15 were considered pre-senescent (young) and >p25 were treated as senescent (old). >p25 cells were confirmed to be senescent by measuring p16 expression and the presence of heterochromatin foci and senescence-associated (SA) β-galactosidase staining (Fig. S3).

SA-β-galactosidase staining

SA-β-galactosidase staining was performed as per manufacturer's protocol from senescence-galactosidase staining kit (Cell Signaling Technology). Briefly, cells were seeded in 6-well tissue culture plates overnight. On the day of assay, cells were washed once with PBS, and fixed with 1× fixative solution (1 mL per well) at room temperature for 15 min. The plate was then washed twice with PBS and stained with 1 mL of β-gal staining solution for overnight at 37°C in a dry non-CO_2 incubator. Images were in nine random fields per condition using light microscopy.

Heterochromatin foci staining

Cells were seeded on coverslips in 6-well tissue culture plates overnight. On the day of staining, cells were washed once with PBS and fixed with cold 4% formaldehyde solution for 15 min at room temperature. The cells were then washed once with cold PBS and mounted on glass slides with Prolong™ Gold Antifade Mount containing DAPI (Thermo Fisher Scientific). There slides were left to dry for overnight in the dark and sealed with clear nail polish. There images were taken using florescent microscope.

Microplate intracellular calcium imaging

Intracellular calcium levels were determined using ratiometric analysis of the calcium binding dye Fura-2-AM with the Molecular Devices FlexStation 3 Multi-mode Microplate Reader. This cell-permeable dye excites at 340 nm when bound to calcium and 380 nm when unbound. Fluorescence intensities were then measured at the emission wavelength of 510 nm. These intensities were represented as a ratio of 340/380 nm.

Cells were seeded on 96-well plates 24 h before performing assay. At the time of assay, media was removed, and cells were incubated with 4 μM Fura-2-AM for 30 min at 37°C. Cells were then washed with 1× HBSS + dextrose three times. Cell culture plate was mounted in a FlexStation plate reader and the treatments were added at specific time points using the Flex mode and fluorescence measurements were obtained.

Microscopic intracellular calcium imaging

Cells were seeded on 6-well plates with cover slips. Cells were incubated with 4 μM Fura-2-AM for 30 min at 37°C in a chamber, washed with 1× HBSS+dextrose without Ca^{2+} three times, and mounted on the microscope. Fluorescence measurements were obtained at 10 readings per minute and analyzed using a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH). At indicated times, solutions on coverslip were exchanged.

Quantitative real-time PCR

RNA was isolated from samples using Trizol (Invitrogen) per manufacturer's instructions. cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with oligo (dT) primers. Real-Time PCR was performed with an Applied Biosystems (AB) 7500 Real-Time Thermocycler with AB SYBR Green. Specific primers were used for each target and the PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 sec, annealing and elongation at 60°C for 1 min. Product specificity was determined by melt curve CT values were normalized to a control housekeeping gene, Actin, and the fold change was found using the ΔΔCT method of analysis. See Table 1 for primer sequences used in the study.

Western blot analysis

Cells were lysed in an appropriate volume of RIPA buffer at pH 7.5 (50 mM Tris-HCl, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 mM Na_3VO_4, and protease inhibitors) for 15 min. Cell debris was pelleted by centrifugation at 15,000 r/min at 4°C and the protein-containing supernatant was analyzed for protein concentration using BCA reagents A and B (Thermo Scientific). Equal amounts of protein were then subjected to polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes followed by overnight incubation with primary antibodies at 4°C. The following day, the membranes were incubated with suitable secondary antibodies at room temperature and the blots were visualized using chemiluminescence.

Statistical analysis

All statistical analyses were performed with the GraphPad Prism 7.0 statistical software package. Statistical significance was assessed using the two-tailed Student's *t* test or one-way ANOVA with Tukey–Kramer post-test. *P* values of <0.05 were considered significant.

Results

Senescence is accompanied by disruption of intracellular Ca^{2+} homeostasis

To define basal levels of intracellular Ca^{2+} in pre-senescent and senescent fibroblasts, we used the fluorescent dye Fura Red/AM whose fluorescence is impeded in the presence of Ca^{2+}.[34] Fura Red intensity profiles, monitored by imaging flow cytometry, revealed that baseline intracellular Ca^{2+} is higher in senescent cells, as observed by reduced fluorescence (Figure 1(a) and (b)) which was reversed in response to low oxygen (3%) (Figure 1(a) and 1(b)). To assess the response of IMR-90 cells to an exogenous stimulus of Ca^{2+}, the relative concentration of intracellular Ca^{2+} was determined using the ratiometric Ca^{2+} binding dye Fura-2. While baseline Ca^{2+} levels remained higher in senescent fibroblasts they failed to respond to addition of extracellular Ca^{2+} (2 mM) in the bath solution when compared to pre-senescent cells (Figure 1(c) and (d)). We next sought to pharmacologically identify the senescence regulated Ca^{2+} channels with 2-APB and SKF-96365. 2-APB is a broad-spectrum compound that both inhibits and activates STIM/Orai-mediated store-operated Ca^{2+} entry (SOCE) channels depending on dose.[35] SKF-96365, though a SOCE inhibitor, also modulates Ca^{2+} entry through certain TRPC channels.[36] Pre-treatment with SKF had no effect on extracellular Ca^{2+}-mediated Ca^{2+} influx in pre-senescent cells while eliciting a sustained Ca^{2+} response in senescent cells (Figure 1(e) and (f)). Pretreatment with 2-APB enhanced Ca^{2+} entry into both pre-senescent and senescent cells, with the pre-senescent cells showing an increased sensitivity to this Ca^{2+} channel modulator (Figure S1). We next performed patch-clamp electrophysiology to determine if SA increases in cytosolic Ca^{2+} are mediated through depletion of SOCE. Addition of BAPTA in the patch pipette

Table 1. Primer sequences used in the study.

Species	Target gene	Specific Primer	
		Sense 5′-3′	Anti-sense 5'-3'
Human	β-Actin	AAAGACCTGTACGCCAACACAGTGCTGTCT	CGTCATACTCCTGCTTGCTGATCCACATCTG
	IL-1α	AACCAGTGCTGCTGAAGGA	TTCTTAGTGCCGTGAGTTTCC
	IL-8	CTCTCTTGGCAGCCTTCCTGATT	AACTTCTCCACAACCCTCTGCAC
	Il-6	CCACACAGACAGCCACTCACC	CTACATTTGCCGAAGAGCCCTC
	CDKN2A (p16)	CTTCGGCTGACTGGCTGG	TCATCATGACCTGGATCGGC
	CDKN1A (p21)	AGTCAGTTCCTTGTGGAGCC	GACATGGCGCCTCCTCTG
	TRPC6	CTTGTGGTCCTTGCTGTTGC	TCTTCCCCATCTTGCTGCAT

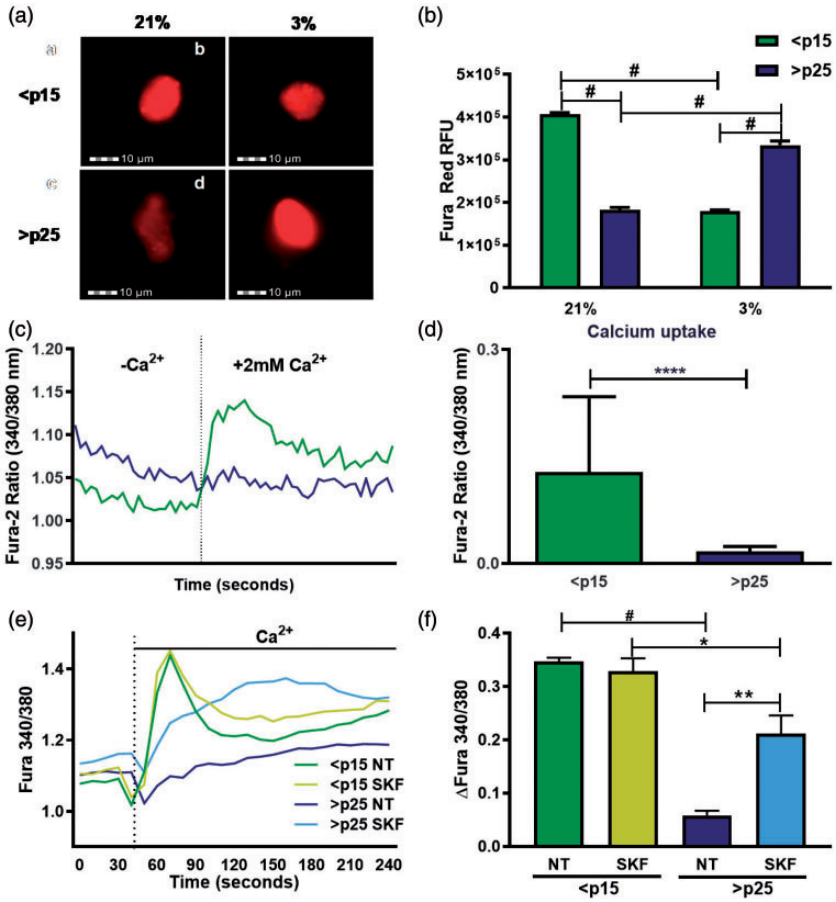

Figure 1. Senescence is accompanied by dysregulation of intracellular Ca^{2+} signaling mechanisms. (a) Pre-senescent (<p15) and senescent (>p25) levels of intracellular Ca^{2+} as measured using the inverse Ca^{2+} fluorescent dye Fura Red as monitored by imaging flow cytometer under ambient (21%) and low (3%) oxygen conditions. 5000 individual cells (events) were imaged. (b) Quantification of mean Fura Red relative fluorescence intensity representing the RFU ± SEM of multiple independent experiments, $n = 5000$ events. (c) Representative ratiometric Fura-2 Ca^{2+} imaging trace of pre-senescent (<p15) and senescent (>p25) fibroblasts treated with an exogenous source of Ca^{2+} (2 mM). At time 40 s bath media was changed to include 2 mM Ca^{2+}. (d) Total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus represented as ΔFura 340/380 ± SEM, $n = 3$. (e) Ratiometric Ca^{2+} imaging of pre-senescent and senescent fibroblasts untreated or treated with 10 μM SKF-96365 15 min before imaging. At time 40 s media was changed to include 2 mM Ca^{2+}. (f) Total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus represented as ΔFura 340/380 ± SEM, $n = 3$. $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.0001$. (A color version of this figure is available in the online journal.)

allows for monitoring the biophysical manifestation of SOCE in the form of I_{CRAC} (Ca^{2+} Release-Activated Ca^{2+} current).[37] BAPTA failed to activate I_{CRAC} in both young and old cell types, while H_2O_2 activated a small inwardly rectifying I_{CRAC}-like current in both cell types with similar magnitudes. This finding suggests that differences in SOCE do not explain the distinct Ca^{2+} entry profiles between pre-senescent and senescent cells (Figure S2).

SA dysregulation of Ca^{2+} is redox-regulated

Several redox sensitive Ca^{2+} channels have been shown to mediate Ca^{2+} influx.[18,38] To demonstrate the redox sensitivity of the Ca^{2+} channel(s) under study, we used the ratiometric dye Fura-2 to determine whether cells respond to a secondary exogenous H_2O_2 stimulus, that has been known to elicit a Ca^{2+} response, in addition to extracellular Ca^{2+}. Exposure to increasing concentration of H_2O_2 (50, 150, and

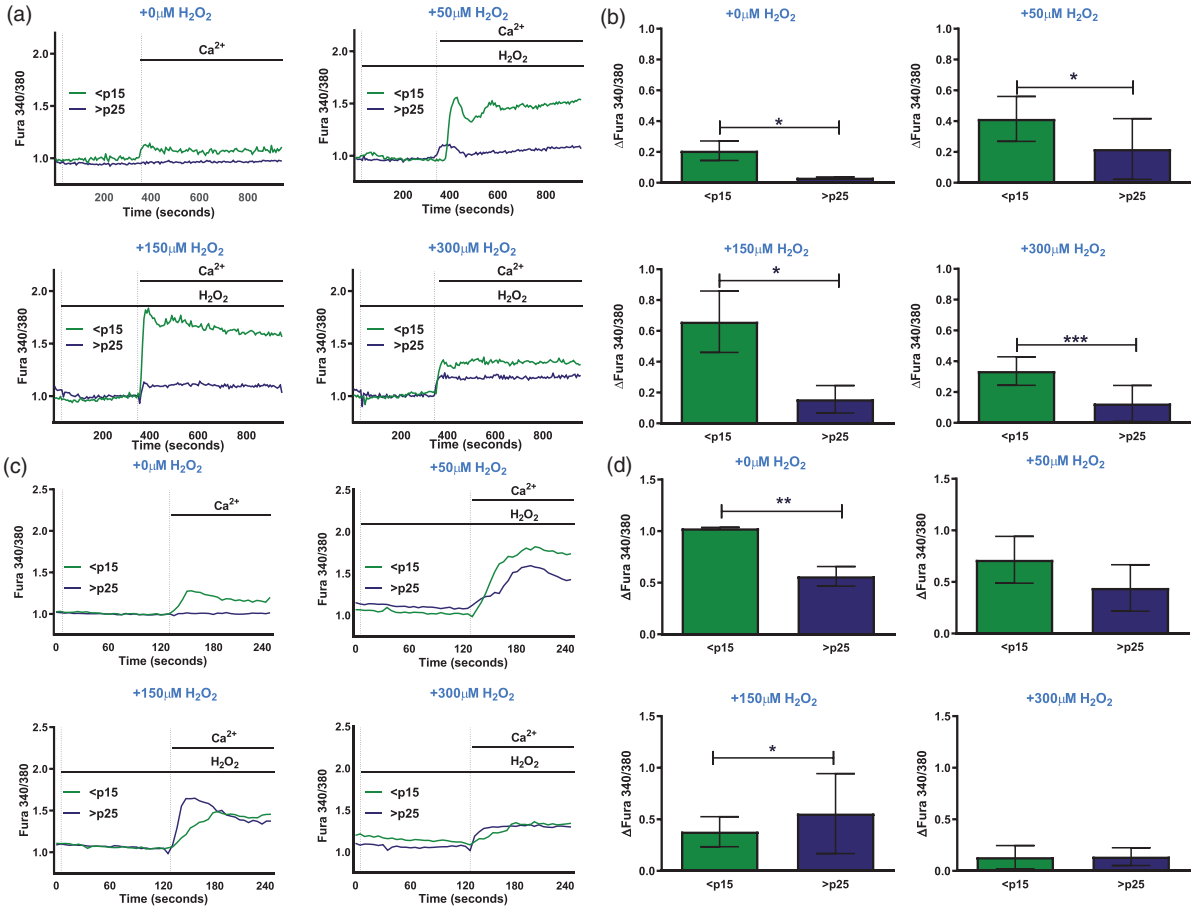

Figure 2. Senescence-associated disruption of Ca^{2+} homeostasis is H_2O_2-regulated. (a) Ratiometric Ca^{2+} imaging of pre-senescent (<p15) and senescent (>p25) fibroblasts with 2 mM Ca^{2+} added at time 330 s and stimulated with increasing H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM. (b) Quantification of total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus with varying H_2O_2 concentrations represented as ΔFura 340/380 ± SEM, $n = 3$. (c) Ratiometric Ca^{2+} imaging of pre-senescent (<p15) and senescent fibroblasts pre-incubated with 200 U/mL recombinant catalase overnight with 2 mM Ca^{2+} added at time 330 s and stimulated with increasing H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM. (d) Quantification of total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus with varying H_2O_2 concentrations represented as ΔFura 340/380 as shown in Figure 1. (A color version of this figure is available in the online journal.)

300 μM) did not stimulate Ca^{2+} entry in the absence of extracellular Ca^{2+} regardless of age (Figure 2(a) and (b)). Upon addition of extracellular Ca^{2+}, pre-senescent cells responded with a robust increase in Ca^{2+} entry that was severely attenuated in senescent cells.

We have previously demonstrated that senescent cells increase steady-state H_2O_2 levels 3.5-fold from 13.7 to 48.6 pM relative to pre-senescent cells.[39] We next determined if the impairment in SA H_2O_2-mediated Ca^{2+} entry is attributed to chronic exposure to elevated H_2O_2. Pre-senescent and senescent cells were treated with 200 U/mL recombinant catalase overnight and the above-mentioned Ca^{2+} traces were repeated. Catalase pre-incubation rescued the inability of the senescent cells to respond to H_2O_2-induced entry of Ca^{2+} (Figure 2(c) and (d)). Together, these results indicate that SA disruption of Ca^{2+} homeostasis is driven by a H_2O_2 sensitive regulatory switch.

TRPC6 is overexpressed in senescence but not functional

TRP Canonical 6 (TRPC6) stands out as a potential redox-regulated Ca^{2+} channel[40] that has pathophysiological implications in age-associated disorders.[41] TRPC3, 6, and 7 channels comprise a subgroup of Ca^{2+}-permeable cation channels within the TRP superfamily and are activated by products of phospholipase C-mediated breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2).[42] Relative to pre-senescent cells, senescent fibroblasts display significant increase in both TRPC6 mRNA and protein (Figure 3(a) and (b)). Next, we tested the functionality of TRPC6 in senescent cells and the ability of the channel to respond to a specific agonist. Hyperforin, a polyprenylated acylphloroglucinol phytochemical, is the key active ingredient in clinically used anti-depressants and the compound selectively activates TRPC6 without stimulating other TRPC channel isoforms.[43] We observed that senescent cells were insensitive to the action of 10 μM hyperforin (Figure 3(c) and (d)) added along with 2 mM Ca^{2+} as compared to pre-senescent cells. However, pre-incubation with 200 U/mL recombinant catalase recovers senescent TRPC6 responses to hyperforin-induced Ca^{2+} entry, further illustrating dysregulation of a redox-sensitive Ca^{2+} channel accompanying the senescent program.

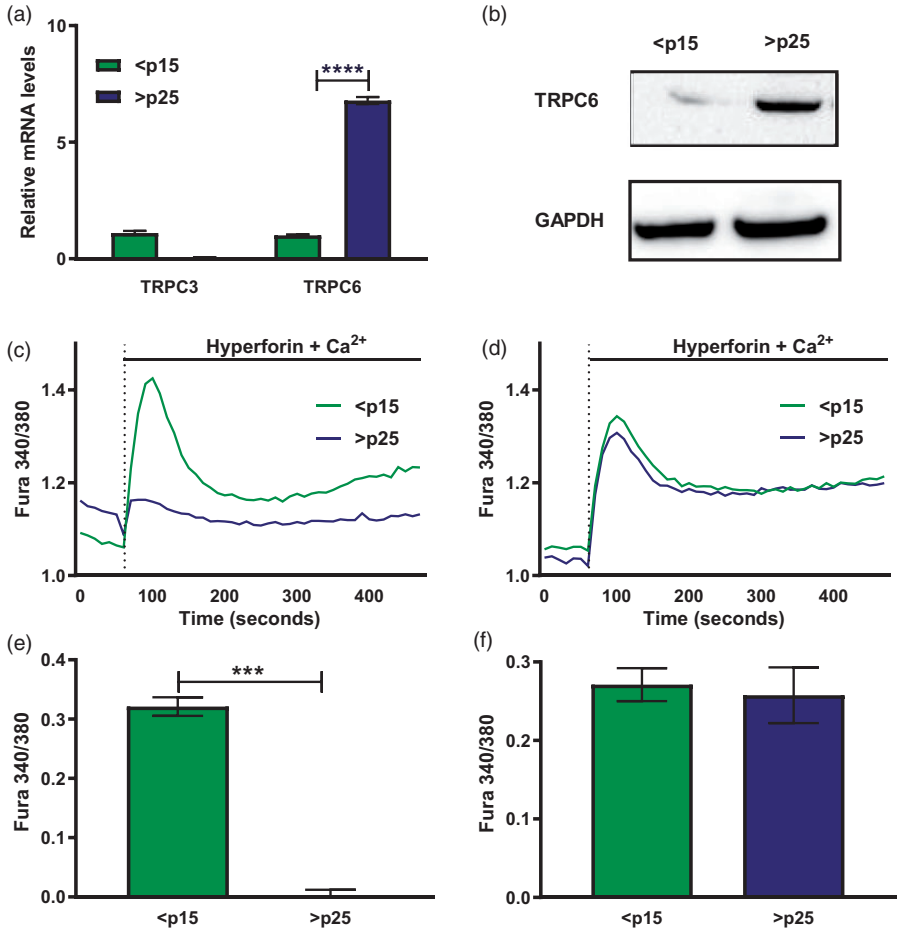

Figure 3. TRPC6 is overexpressed during senescence and its agonist unresponsiveness is restored by catalase pretreatment. Relative mRNA levels of TRPC3 and TRPC6 channels in pre-senescent (<p15) and senescent (>p25) cells. (b) TRPC6 and GAPDH protein abundance as measured from cell lysates of pre-senescent and senescent cells. (c) Ratiometric Ca^{2+} imaging of pre-senescent and senescent fibroblasts with 10 μM hyperforin and 2 mM Ca^{2+} added at time 60 s. (d) Quantification of total change in intracellular Ca^{2+} after addition of 10 μM hyperforin and 2 mM Ca^{2+} stimulus represented as ΔFura 340/380 ± SEM, $n = 3$. (e) Ratiometric Ca^{2+} imaging of pre-senescent and senescent fibroblasts pre-incubated with 200 U/mL recombinant catalase overnight with 10 μM hyperforin and 2 mM Ca^{2+} added at time 60 s (f) Quantification of total change in intracellular Ca^{2+} after addition of 10 μM hyperforin and 2 mM Ca^{2+} stimulus represented as shown in Figure 1. (A color version of this figure is available in the online journal.)

SKF reverses Ca^{2+} dysregulation and suppresses SASP

To test the role of the impaired Ca^{2+} signaling in senescence and SASP, we took advantage of the ability of SKF to restore SA Ca^{2+} entry (Figure 1(e)) and determine its impact on H_2O_2-mediated Ca^{2+} mobilization and on SASP gene expression. Upon pre-treatments with 10 μM SKF for 15 min, we found that senescent fibroblasts retrieved their ability to mobilize Ca^{2+} in response to H_2O_2 (Figure 4(a) and (b)). Comparable to the effects of catalase in rescuing the ability of senescent cells to increase cytosolic Ca^{2+} in response to H_2O_2 (Figure 2), SKF treatments restored Ca^{2+} mobilization capacity with different concentrations of H_2O_2 (50, 150, and 300 μM). Additionally, IMR-90 fibroblasts were treated with 10 μM SKF for 24 h to monitor TRPC6 mRNA levels (Figure 4(c)). We found that SKF inhibited SA TRPC6 expression as well as the mRNA levels of the cell cycle inhibitor p16 and key SASP factors IL-8, IL-6, IL-1α, and MMP-1 (Figure 4(c)). Taken together, these data indicate that restoring Ca^{2+} entry with SKF limits SASP.

mTOR regulates TRPC6 and SASP

mTOR complex 2 (mTORC2) has been shown to be a regulator of TRPC6 channel expression and activity in podocytes[44] and is thought to be essential for maintenance of the senescent state[45] Our findings indicate that restoration of SA extracellular Ca^{2+} mobilization can limit SASP. We next determined whether mTOR inhibition which blocks SASP,[46] modulates calcium homeostasis ether basally or in response to H_2O_2 treatment in senescent IMR-90 fibroblasts. Exposure of senescent cells to rapamycin, an inhibitor of mTORC1, or Ku0063794, a dual inhibitor of mTORC1 and mTORC2, for 24 h augmented the ability of senescent cells to mobilize cytosolic Ca^{2+} after stimulation with 2 mM Ca^{2+} (Figure 5(a) and (b)). Rapamycin and Ku0063794 also rescued the ability of the senescent cells to respond to increasing concentrations of H_2O_2 at 50, 150, and 300 μM (Figure 5(a) and (b)). We further monitored the impact of rapamycin and Ku0063794 on SASP transcript levels and observed reduction in TRPC6, p16, IL-8, IL-6, IL-1α, and MMP-1 (Figure 5(c)). Collectively, these data suggest a

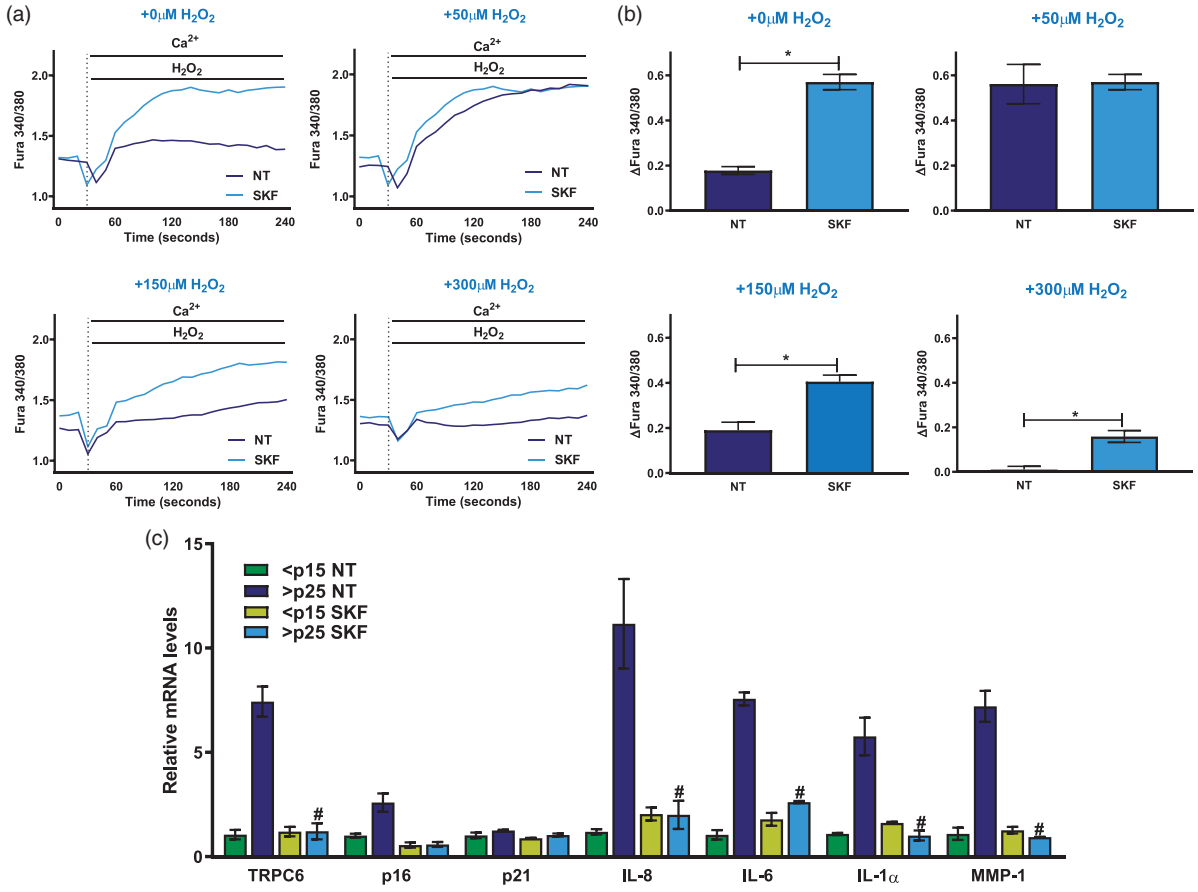

Figure 4. SKF reverses senescence Ca^{2+} dysregulation and suppresses SASP. (a) Ratiometric Ca^{2+} imaging of senescent fibroblasts with 2 mM Ca^{2+} added at time 40 s following 15 min treatment with or without 10 μM SKF-96365 for and stimulated with increasing H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM. (b) Total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus with varying H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM as shown in Figure 1. (c) Relative mRNA levels of key senescence and SASP components in pre-senescent and senescent cells as measured by real-time PCR. Results expressed as mean ± SEM and statistically compared against >p25 NT, $n = 3$. $^{*}P \leq 0.05$, $^{\#}P < 0.0001$. (A color version of this figure is available in the online journal.)

critical role for mTOR signaling in regulating SASP and TRPC6 transcripts levels as well as the SA disruption in Ca^{2+} homeostasis.

Discussion

Disturbances in intracellular calcium homeostasis have been well established in physiological senescence-associated processes including cell proliferation, autophagy, apoptosis, and necrosis.[47,48] Ca^{2+} acts as an essential second messenger for cell-to-cell communication and for driving intracellular processes. The role of Ca^{2+} dysregulation in physiological and organ-specific aging processes has been delineated.[26,49,50] Elevated intracellular calcium levels have been observed in response to several forms of senescence-inducing stress (telomere shortening, oncogene activation, or oxidative stress) and are orchestrated through plasma membrane Ca^{2+} channels or by Ca^{2+} release from the endoplasmic reticulum.[19] For example, Retinoid X receptor alpha (RXRA) is also been shown to regulate Ca^{2+} signaling through inositol 1, 4, 5-triphosphate receptor type 2 (ITPR2), an endoplasmic reticulum calcium release channel, and mitochondrial calcium uniporter (MCU). Increased ROS production and DNA damage via ITPR2-MCU calcium signaling pathway, triggering senescence via p53 activation are observed in RXRA knocked down human lung fibroblasts, whereas RXRA overexpression can delay replicative senescence.[51] Ca^{2+} entry through non-selective cation channel, transient receptor potential vanilloid 4 (TRPV4) can lead to regulatory volume decrease (RVD) to counteract the increase of cell volume, one of the prominent features of senescent cells. Cells lacking TRPV4 are not able to engage RVD.[52] In addition to TRPV4, TRPM7 channel can also be activated by membrane swelling or osmosis swelling.[53] TRPC7 is showed to involved in initiation of UVB-induced aging and TRPC7 knocked down can suppress the UVB-induced aging processes.[54] Mouse vascular endothelial cells lacking TRPC5 display reduced β-galactosidase staining, implicating in the involvement of TRPC5 in development of senescence.[55]

Our findings support the possibility that the senescence program is in part controlled by disruptions in normal Ca^{2+} signaling as evidenced by: (i) alterations in TRPC channel expression; ii) redox- and mTOR-dependent disruption in Ca^{2+} homeostasis, and (iii) SASP inhibition through restoration of Ca^{2+} entry. Here, we establish that senescent fibroblasts display a basal increase in intracellular Ca^{2+} but fail to respond to exogenous Ca^{2+} addition. Several studies

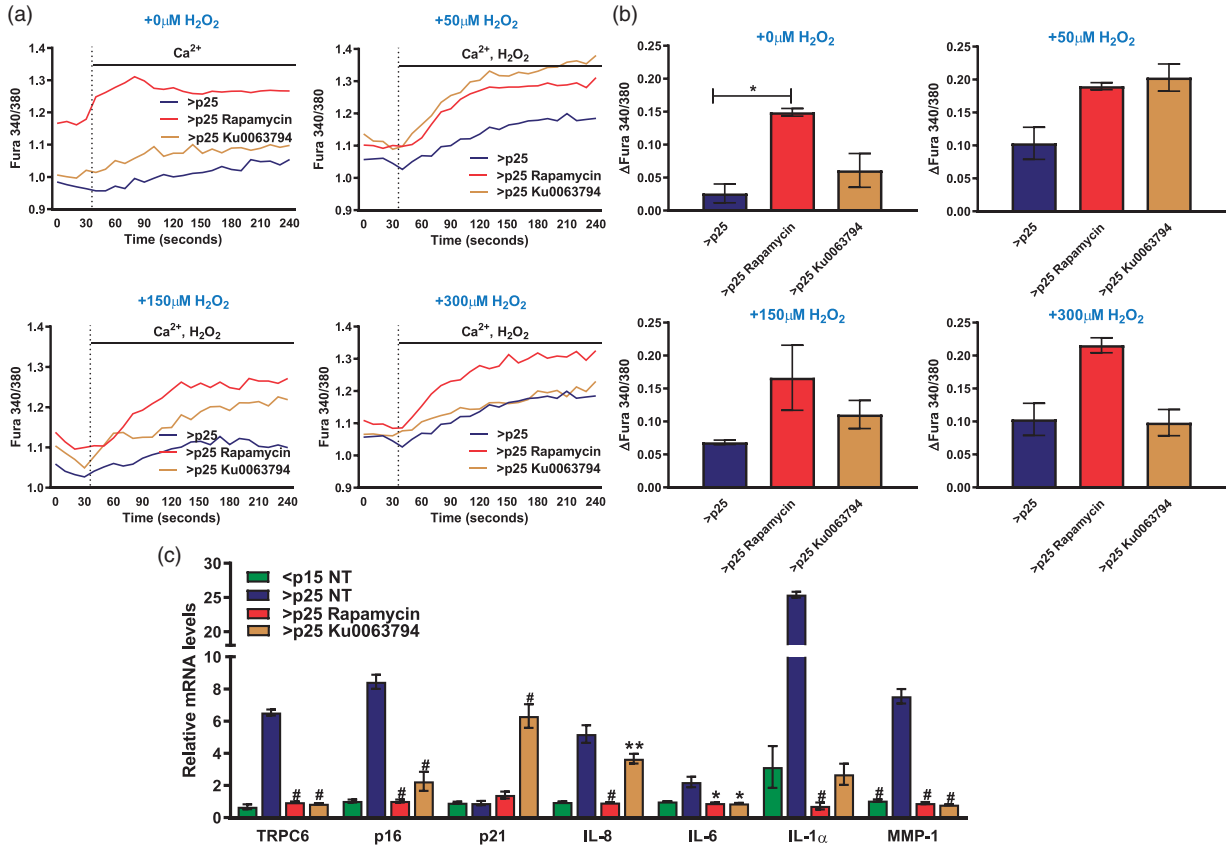

Figure 5. mTOR enhances Ca^{2+} entry and limits replicative SASP and TRPC6 expression. Ratiometric Ca^{2+} imaging of senescent fibroblasts with 2 mM Ca^{2+} added at time 40 s previously untreated or treated with 25 nM rapamycin or 10 μM Ku0063794 and stimulated with increasing H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM. (b) Total change in intracellular Ca^{2+} after addition of 2 mM Ca^{2+} stimulus with varying H_2O_2 concentrations of 0 μM, 50 μM, 150 μM, and 300 μM as shown in Figure 1. (c) Relative mRNA levels of key senescence and SASP components in untreated fibroblasts and senescent (>p25) fibroblasts pre-incubated with 25 nM rapamycin or 10 μM Ku0063794 for 24 has measured by real-time PCR. Results expressed as mean ± SEM and statistically compared against >p25 NT, $n = 3$. *$P \leq 0.05$, **$P < 0.01$, #$P < 0.0001$. (A color version of this figure is available in the online journal.)

point to persistently elevated intracellular Ca^{2+} levels in aged cells as compared to young cells, primarily in neuronal cells.[56–58] We propose that the observed increase in basal Ca^{2+} limits further cellular responses to increases in extracellular Ca^{2+}. These and other findings have been taken to justify a prevalent "calcium overload" hypothesis postulating that Ca^{2+} overload in old cells arising from oxidative stress limits functionality of Ca^{2+} signaling.[18] Increased Ca^{2+} load has been shown to promote IL-1α processing and functionality, initiating the SASP signaling cascade.[26] Here we demonstrate that senescence is accompanied by a defect in extracellular Ca^{2+} mobilization, that is coupled to increases in TRPC6 and SASP gene expression.

The TRPC channels constitute a family of seven different members, TRPC1-7, of physiological and pathophysiological importance.[59,60] TRPC6 expression has been described in selective age-related maladies including vasospastic disorders, hypertension, Alzheimer's disease, and cardiac hypertrophy.[41,61–63] H_2O_2 has been conclusively proven to activate TRP channels,[17,38] including TRPC6, and may govern a novel mechanism for agonist-initiated cellular responses driven by the channel. We provide evidence that hyperforin responsiveness of TRPC6 is lost during senescence and restored by efficient enzymatic H_2O_2 removal by catalase. Catalase pretreatment also rescues SA loss of H_2O_2-Ca^{2+} entry. However, whether rescue of both hyperforin and H_2O_2-dependent Ca^{2+} entry occurs through TRPC6 remains to be established. The recovery of H_2O_2-induced Ca^{2+} influx by catalase illustrates a dual role for H_2O_2 in both activating and limiting TRPC6 function. The effect of H_2O_2 is likely reliant on abundance and duration of exposure to this ubiquitous second messenger. While ROS serves to activate TRPC6 acutely (minutes),[64] prolonged exposure, as with the case in several oxidative stress-propagated chronic pathologies, can suppress TRPC6 channel expression.[65] TRPC6 activity has been reported to be controlled by diverse modalities including diacylglycerol (DAG), the plant extract hyperforin and H_2O_2. H_2O_2 activates TRPC6 by modifying thiol groups promoting its trafficking to the cell surface, conferring DAG sensitivity and channel activation.[40] We propose that the TRPC6 channel is overexpressed during senescence and its activity is compromised by increases in steady-state H_2O_2 production as demonstrated by a lack of senescent cell response to TRPC6-activating hyperforin (Figure 3). Though hyperforin is touted to possess free radical scavenging property,[66] hyperforin alone is not sufficient to restore the Ca^{2+} mobilizing ability of senescent cells. Given the role of TRPC6 in driving disease pathology,

identification of the mechanisms dictating chronic regulation of TRPC6 by H_2O_2 has potential clinical implications.

The age-associated phenotypic shift that that heightens SA Ca^{2+} levels has been shown to drive the processing of IL-1α and its ability to propagate the SASP.[26] We have identified TRPC6 as a potential participant in SA Ca^{2+} dysregulation and SASP activation. While SKF-96365 is often used to inhibit Store-operated Ca^{2+} entry and TRPC channel activity, our findings indicate that SKF enhances SA Ca^{2+} entry and limits the expression of the senescence marker p16 and SASP genes IL-6, IL-8, IL-1α and MMP-1 and TRPC6 itself. How SKF enhances senescence Ca^{2+} entry is not clear but may involve activation of reverse-mode of Ca^{2+} entry through the sodium-calcium exchange, NCX-1, as has been reported in cardiac fibroblasts and glioblastoma cells.[67,68] Recent studies have also shown the loss of the sodium channel, SCN9a, which regulates plasma membrane depolarization allows cellular escape from oncogene-induced senescence.[35] Maintenance of plasma membrane potential is reliant on coordinate regulation of numerous ion channels and together these observations suggest that the dysregulation of ion channel homeostasis impacts downstream signaling which results in the amplification of SA gene expression.mTOR signaling also contributes to regulation of TRPC6 channel expression and activity[44,69] as well as SASP gene expression.[46,70] Blocking TRPC6 can promote H_2O_2-mediated autophagy through activation of P13K/Akt/mTOR pathway.[71] mTORC2 inhibition has been shown to be responsible for limiting TRPC6 expression in podocytes which are critical for the maintenance of glomerular filtration barrier.[44] While mTOR inhibition in models of oncogene or ionizing radiation-induced senescence restricts SASP gene expression by both limiting the translation of the SASP regulator IL-1α[70] and through mRNA destabilization of SASP transcripts.[46] In our hands, both mTOR inhibitors, rapamycin and ku0063794, serve to restore Ca^{2+} entry and limit TRPC6 and SASP gene expression resulting from replicative senescence (Figure 5). Inhibition of replicative SASP gene expression by mTOR blockade likely occurs through a similar signaling that restricts IL-1α production[70] and promotes SASP transcript destabilization.[46] Inhibition of SA TRPC6 expression by mTOR might be attributed to the presence of a number of AUUUA mRNA destabilizing elements in its 3-untranslated region (3′-UTR) which have been shown to play an important role in SASP mRNA stabilization.[46] Analysis of the TRPC6 3′-UTR with the ARE score tool[72] identified the presence of nine AUUUA pentamers and an ARE score of 11.1, a score which is comparable to many of the labile SASP transcripts. We propose that mTOR elicits regulatory effects on SASP by modulating Ca^{2+} homeostasis through TRPC6 channel expression. The idea that the mTOR inhibitors also rescue peroxide-induced Ca^{2+} responses in senescence alludes to an antioxidant-like or antioxidant-promoting nature of these compounds that function to provide defense against cellular oxidative stress. This study furthers our understanding of how SA shifts in steady-state H_2O_2 levels serve to disrupt Ca^{2+} homeostasis and SASP. As TRP channels emerge as targets of pharmacologic intervention for numerous disease pathologies, it is exciting to speculate that the effect of TRP interventions may be attributed in part to inhibition of the SASP.

Future Directions

Common age-related metabolic diseases include type-2 diabetes mellitus (T2DM), obesity, atherosclerosis, sarcopenia, and macular degeneration. Since mitochondria are the master regulators of cellular metabolism, it is not surprising that the key underlying factor among these metabolism diseases is mitochondrial dysfunction.[73] The quality and quantity of mitochondria are essential to maintain the energetic balance to meet tissue-specific energy demand, as well as to maintain healthy ROS production for signaling purposes. Mitochondria maintain their health through dynamic processes, including mitochondrial fusion, which helps in fusing mitochondria to enhance the energy production, and mitochondrial fission, which helps segregating the damaged mitochondria.[74,75] Mitochondrial matrix Ca^{2+} is an important factor for regulating the production of reducing enzymes essential for electron transport chain (ETC), involved in retaining proton gradient required for ATP production.[76] Ca^{2+} transfer from endoplasmic reticulum to mitochondrial must be tightly regulated as excess Ca^{2+} can disturb oxidative phosphorylation, increasing the release of ROS, which can lead to cellular damage observed in both aging and neurodegenerative diseases.[77] Ca^{2+} overload due to increased uptake of calcium as a result of enhanced ER-mitochondrial tethering is observed in aged endothelial cells.[78] In addition, increased H_2O_2 as the result of calcium overload is also observed in aged muscle tissues.[79] The accumulation of Ca^{2+} in mitochondrial through mitochondrial calcium uniporter (MCU) from ER can be triggered by ITPR2 during replicative senescence, which can lead to drop in membrane potential and increased ROS.[23] With heavy crosstalk between mitochondrial calcium signaling and senescence, it will be interesting to explore the involvement of mitochondrial dynamics in mTOR-dependent regulation of TRPC6 and cellular senescence.

Authors' contributions: AC collected and analyzed data. AC and ML contributed equally to the preparation of the manuscript. All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript and S.A.T for providing critical reagents.

ACKNOWLEDGEMENTS

The authors thank Mohamed Trebak from Penn State for his helpful guidance and insight.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was generously supported in part by the SUNY Polytechnic Institute Research Seed Grant Program, in part by National Institute of Health R01 [grant number GM125870] to S.A.T.

ORCID iDs

May Y Lee https://orcid.org/0000-0002-3432-4279
J Andrés Melendez https://orcid.org/0000-0001-8021-3097

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961;**25**:585–621
2. Courtois-Cox S, Jones SL, Cichowski K. Many roads lead to oncogene-induced senescence. *Oncogene* 2008;**27**:2801–9
3. Liu X, Ling Ding J, Meng L. Hua Oncogene-induced senescence: a double edged sword in cancer. *Acta Pharmacol Sin* 2018;**39**:1553–8
4. Wickens AP. Ageing and the free radical theory. *Respir Physiol* 2001;**128**:379–91
5. McHugh D, Gil J. Senescence and aging: causes, consequences, and therapeutic avenues. *J Cell Biol* 2018;**217**:65–77
6. Harley BC, Futcher BA, Greider WC. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;**345**:458–60
7. de Magalhães JP, Passos JF. Stress, cell senescence and organismal ageing. *Mech Age Dev* 2018;**170**:2–9
8. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, VanSteeg H, Dollé MET, Hoeijmakers JHJ, DeBruin A, Hara E, Campisi J. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* 2014;**31**:722–33
9. Yun MH. Cellular senescence in tissue repair: every cloud has a silver lining. *Int J Dev Biol* 2018;**62**:591–604
10. Jun J, Il Lau LF. Cellular senescence controls fibrosis in wound healing. *Aging* 2010;**2**:627–31
11. Muñ Oz-Espín D, Cañ Amero M, Maraver A, Gó Mez-Ló Pez G, Contreras J, Murillo-Cuesta S, Rodríguez-Baeza A, Varela-Nieto I, Ruberte J, Collado M, Serrano M. Programmed cell senescence during mammalian embryonic development. *Cell* 2013;**155**:1104–18
12. Storer M, Mas A, Robert-Moreno A, Pecoraro M, Ortells MC, Di Giacomo V, Yosef R, Pilpel N, Krizhanovsky V, Sharpe J, Keyes WM. Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* 2013;**155**:1119–30
13. Malaquin N, Martinez A, Rodier F. Keeping the senescence secretome under control: molecular reins on the senescence-associated secretory phenotype. *Exp Gerontol* 2016;**82**:39–49
14. Jones DP. Redox theory of aging. *Redox Biol* 2015;**5**:71–9
15. Chandrasekaran A, Idelchik M, del PS, Melendez JA. Redox control of senescence and age-related disease. *Redox Biol* 2017;**11**:91–102
16. Clapham DE. Calcium signaling. *Cell* 2007;**131**:1047–58
17. Ogawa N, Kurokawa T, Mori Y. Sensing of redox status by TRP channels. *Cell Calcium* 2016;**60**:115–22
18. Nguyena HT, Sawmillera DR, Markova O, Chen M. Elevated [Ca2+]i levels occur with decreased calpain activity in aged fibroblasts and their reversal by energy-rich compounds: new paradigm for ALZHEIMER'S disease prevention. *J Alzheimers Dis* 2013;**37**:835–48
19. Martin N, Bernard D. Calcium signaling and cellular senescence. *Cell Calcium* 2018;**70**:16–23
20. Putney JW, Tomita T. Phospholipase C signaling and calcium influx. *Adv Biol Regul* 2012;**52**:152–64
21. Taylor CW, Tovey SC. IP(3) receptors: toward understanding their activation. *Cold Spring Harb Perspect Biol* 2010;**2**:a004010
22. Taylor CW. Regulation of IP3 receptors by cyclic AMP. *Cell Calcium* 2017;**63**:48–52
23. Wiel C, Lallet-Daher H, Gitenay D, Gras B, Le Calvé B, Augert A, Ferrand M, Prevarskaya N, Simonnet H, Vindrieux D, Bernard D. Endoplasmic reticulum calcium release through ITPR2 channels leads to mitochondrial calcium accumulation and senescence. *Nat Commun* 2014;**5**:3792
24. Yee NS, Brown RD, Lee MS, Zhou W, Jensen C, Gerke H, Yee RK. TRPM8 ion channel is aberrantly expressed and required for preventing replicative senescence in pancreatic adenocarcinoma: potential role of TRPM8 as a biomarker and target. *Cancer Biol Ther* 2012;**13**:592–9
25. Yee NS, Zhou W, Lee M, Yee RK. Targeted silencing of TRPM7 ion channel induces replicative senescence and produces enhanced cytotoxicity with gemcitabine in pancreatic adenocarcinoma. *Cancer Lett* 2012;**318**:99–105
26. McCarthy DA, Clark RR, Bartling TR, Trebak M, Melendez JA. Redox-control of the senescence regulator interleukin-1 α and the secretory phenotype. *J Biol Chem* 2013;**288**:32149–59
27. Xu S, Cai Y, Wei Y. mTOR signaling from cellular senescence to organismal aging. *Aging Dis* 2014;**5**:263–73
28. Ziegler DV, Wiley CD, Velarde MC. Mitochondrial effectors of cellular senescence: beyond the free radical theory of aging. *Aging Cell* 2015;**14**:1–7
29. Correia-Melo C, Marques FD, Anderson R, Hewitt G, Hewitt R, Cole J, Carroll BM, Miwa S, Birch J, Merz A, Rushton MD, Charles M, Jurk D, Tait SW, Czapiewski R, Greaves L, Nelson G, Bohlooly -Y, M, Rodriguez-Cuenca S, Vidal-Puig A, Mann D, Saretzki G, Quarato G, Green DR, Adams PD, Zglinicki T, Korolchuk VI, Passos JF. Mitochondria are required for pro-ageing features of the senescent phenotype. *EMBO J* 2016;**35**:724–42
30. Nacarelli T, Azar A, Sell C. Mitochondrial stress induces cellular senescence in an mTORC1-dependent manner. *Free Radic Biol Med* 2016;**95**:133–54
31. Herranz N, Gallage S, Gil J. TORn about SASP regulation. *Cell Cycle* 2015;**14**:3771–2
32. Decuypere JP, Kindt D, Luyten T, Welkenhuyzen K, Missiaen L, De Smedt H, Bultynck G, Parys JB. mTOR-Controlled autophagy requires intracellular Ca2+ signaling. *PLoS One* 2013;**8**:e61020
33. Martín-Cano FE, Camello-Almaraz C, Hernandez D, Pozo MJ, Camello PJ. mTOR pathway and Ca^{2+} stores mobilization in aged smooth muscle cells. *Aging* 2013;**5**:339–46
34. Lipp P, Niggli E. Ratiometric confocal Ca(2+)-measurements with visible wavelength indicators in isolated cardiac myocytes. *Cell Calcium* 1993;**14**:359–72
35. Warnier M, Flaman J-M, Chouabe C, Wiel C, Gras B, Griveau A, Blanc E, Foy J-P, Mathot P, Saintigny P, Van Coppenolle F, Vindrieux D, Martin N, Bernard D. The SCN9A channel and plasma membrane depolarization promote cellular senescence through Rb pathway. *Aging Cell* 2018;**17**:e12736
36. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol* 2006;**68**:619–47
37. Hogan PG, Rao A. Dissecting ICRAC, a store-operated calcium current. *Trends Biochem Sci* 2007;**32**:235–45
38. Kozai D, Ogawa N, Mori Y. Redox regulation of transient receptor potential channels. *Antioxid Redox Signal* 2014;**21**:971–86
39. Dasgupta J, Kar S, Remmen H, Van Melendez JA. Age-dependent increases in interstitial collagenase and MAP kinase levels are exacerbated by superoxide dismutase deficiencies. *Exp Gerontol* 2009;**44**:503–10
40. Graham S, Ding M, Ding Y, Sours-Brothers S, Luchowski R, Gryczynski Z, Yorio T, Ma H, Ma R. Canonical transient receptor potential 6 (TRPC6), a redox-regulated cation channel. *J Biol Chem* 2010;**285**:23466–76
41. Erac Y, Selli C, Kosova B, Akcali KC, Tosun M. Expression levels of TRPC1 and TRPC6 ion channels are reciprocally altered in aging rat aorta: implications for age-related vasospastic disorders. *Age* 2010;**32**:223–30

42. Trebak M, Vazquez G, Bird GSJ, James WP, Jr. The TRPC3/6/7 subfamily of cation channels. *Cell Calcium* 2003;**33**:451–61
43. Leuner K, Kazanski V, Muller M, Essin K, Henke B, Gollasch M, Harteneck C, Muller WE. Hyperforin a key constituent of St. John's Wort specifically activates TRPC6 channels. *FASEB J* 2007;**21**:4101–11
44. Ding F, Zhang X, Li X, Zhang Y, Li B, Ding J. Mammalian target of rapamycin complex 2 signaling pathway regulates transient receptor potential cation channel 6 in podocytes. *PLoS One* 2014;**9**:e112972
45. Tominaga-Yamanaka K, Abdelmohsen K, Martindale JL, Yang X, Taub DD, Gorospe M. NF90 coordinately represses the senescence-associated secretory phenotype. *Aging* 2012;**4**:695–708
46. Herranz N, Gallage S, Mellone M, Wuestefeld T, Klotz S, Hanley CJ, Raguz S, Acosta JC, Innes AJ, Banito A, Georgilis A, Montoya A, Wolter K, Dharmalingam G, Faull P, Carroll T, Martínez-Barbera JP, Cutillas P, Reisinger F, Heikenwalder M, Miller RA, Withers D, Zender L, Thomas GJ, Gil J. mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat Cell Biol* 2015;**17**:1205–17
47. Zhivotovsky B, Orrenius S. Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium* 2011;**50**:211–21
48. Pinto MCX, Kihara AH, Goulart VAM, Tonelli FMP, Gomes KN, Ulrich H, Resende RR. Calcium signaling and cell proliferation. *Cell Signal* 2015;**27**:2139–49
49. Ureshino RP, Rocha KK, Lopes GS, Bincoletto C, Smaili SS. Calcium signaling alterations, oxidative stress, and autophagy in aging. *Antioxid Redox Signal* 2014;**21**:123–37
50. Borodkina AV, Shatrova AN, Deryabin PI, Griukova AA, Abushik PA, Antonov SM, Nikolsky NN, Burova EB. Calcium alterations signal either to senescence or to autophagy induction in stem cells upon oxidative stress. *Aging* 2016;**8**:3400–18
51. Ma X, Warnier M, Raynard C, Ferrand M, Kirsh O, Defossez PA, Martin N, Bernard D. The nuclear receptor RXRA controls cellular senescence by regulating calcium signaling. *Aging Cell* 2018;**17**:e12831
52. Becker D, Blase C, Bereiter-Hahn J, Jendrach M. TRPV4 exhibits a functional role in cell-volume regulation. *J Cell Sci* 2005;**118**:2435–40
53. Numata T, Shimizu T, Okada Y. Direct mechano-stress sensitivity of TRPM7 channel. *Cell Physiol Biochem* 2007;**19**:1–8
54. Hsu WL, Tsai MH, Wu CY, Liang JL, Lu JH, Kahle JS, Yu HS, Yen CJ, Yen CT, Hsieh YC, Huang YY, Lin LC, Tsai TF, Chen CH, Yoshioka T. Nociceptive transient receptor potential canonical 7 (TRPC7) mediates aging-associated tumorigenesis induced by ultraviolet B. *Aging Cell* 2020;**19**:1
55. Li Z, Guo G, Wang H, Si X, Zhou G, Xiong Y, Li S, Dai R, Yang C. TRPC5 channel modulates endothelial cells senescence. *Eur J Pharmacol* 2017;**802**:27–35
56. Murchison D, Griffith WH. Calcium buffering systems and calcium signaling in aged rat basal forebrain neurons. *Aging Cell* 2007;**6**:297–305
57. Övey IS, Naziroğlu M. Homocysteine and cytosolic GSH depletion induce apoptosis and oxidative toxicity through cytosolic calcium overload in the hippocampus of aged mice: involvement of TRPM2 and TRPV1 channels. *Neuroscience* 2015;**284**:225–33
58. Ishii T, Takanashi Y, Sugita K, Miyazawa M, Yanagihara R, Yasuda K, Onouchi H, Kawabe N, Nakata M, Yamamoto Y, Hartman PS, Ishii N. Endogenous reactive oxygen species cause astrocyte defects and neuronal dysfunctions in the hippocampus: a new model for aging brain. *Aging Cell* 2017;**16**:39–51
59. Vazquez G, Wedel BJ, Aziz O, Trebak M, Putney JW. The mammalian TRPC cation channels. *Biochim Biophys Acta* 2004;**1742**:21–36
60. Putney JW. The enigmatic TRPCs: multifunctional cation channels. *Trends Cell Biol* 2004;**14**:282–6
61. Toth P, Tucsek Z, Sosnowska D, Gautam T, Mitschelen M, Tarantini S, Deak F, Koller A, Sonntag WE, Csiszar A, Ungvari Z. Age-related autoregulatory dysfunction and cerebromicrovascular injury in mice with angiotensin II-induced hypertension. *J Cereb Blood Flow Metab* 2013;**33**:1732–42
62. Ding H-Y, Ma H-X. Significant roles of anti-aging protein klotho and fibroblast growth factor23 in cardiovascular disease. *J Geriatr Cardiol* 2015;**12**:439–47
63. Zhang H, Sun S, Wu L, Pchitskaya E, Zakharova O, Fon Tacer K, Bezprozvanny I. Store-operated calcium channel complex in postsynaptic spines: a new therapeutic target for Alzheimer's disease treatment. *J Neurosci* 2016;**36**:11837–50
64. Ding Y, Winters A, Ding M, Graham S, Akopova I, Muallem S, Wang Y, Hong JH, Gryczynski Z, Yang S-H, Birnbaumer L, Ma R. Reactive oxygen species-mediated TRPC6 protein activation in vascular myocytes, a mechanism for vasoconstrictor-regulated vascular tone. *J Biol Chem* 2011;**286**:31799–809
65. Graham S, Gorin Y, Abboud HE, Ding M, Lee DY, Shi H, Ding Y, Ma R. Abundance of TRPC6 protein in glomerular mesangial cells is decreased by ROS and PKC in diabetes. *Am J Physiol Cell Physiol* 2011;**301**:C304–C315
66. Meinke MC, Schanzer S, Haag SF, Casetti F, Müller ML, Wölfle U, Kleemann A, Lademann J, Schempp CM. In vivo photoprotective and anti-inflammatory effect of hyperforin is associated with high antioxidant activity in vitro and ex vivo. *Eur J Pharm Biopharm* 2012;**81**:346–50
67. Ikeda K, Nakajima T, Yamamoto Y, Takano N, Tanaka T, Kikuchi H, Oguri G, Morita T, Nakamura F, Komuro I. Roles of transient receptor potential canonical (TRPC) channels and reverse-mode Na+/Ca2+ exchanger on cell proliferation in human cardiac fibroblasts: effects of transforming growth factor β1. *Cell Calcium* 2013;**54**:213–25
68. Song M, Chen D, Yu SP. The TRPC channel blocker SKF 96365 inhibits glioblastoma cell growth by enhancing reverse mode of the Na(+)/Ca(2+) exchanger and increasing intracellular Ca(2+). *Br J Pharmacol* 2014;**171**:3432–47
69. Zhang HT, Wang WW, Ren LH, Zhao XX, Wang ZH, Zhuang DL, Bai YN. The mTORC2/akt/NFKB Pathway-Mediated activation of TRPC6 participates in Adriamycin-Induced podocyte apoptosis. *Cell Physiol Biochem* 2016;**40**:1079–93
70. Laberge R-M, Sun Y, Orjalo AV, Patil CK, Freund A, Zhou L, Curran SC, Davalos AR, Wilson-Edell KA, Liu S, Limbad C, Demaria M, Li P, Hubbard GB, Ikeno Y, Javors M, Desprez P-Y, Benz CC, Kapahi P, Nelson PS, Campisi J. MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nat Cell Biol* 2015;**17**:1049–61
71. Hou X, Xiao H, Zhang Y, Zeng X, Huang M, Chen X, Birnbaumer L, Liao Y. Transient receptor potential channel 6 knockdown prevents apoptosis of renal tubular epithelial cells upon oxidative stress via autophagy activation. *Cell Death Dis* 2018;**9**:1–15
72. Spasic M, Friedel CC, Schott J, Kreth J, Leppek K, Hofmann S, Ozgur S, Stoecklin G. Genome-wide assessment of AU-rich elements by the AREScore algorithm. *PLoS Genet* 2012;**8**:e1002433
73. Natarajan V, Chawla R, Mah T, Vivekanandan R, Tan SY, Sato PY, Mallilankaraman K. Mitochondrial dysfunction in age-related metabolic disorders. *Proteomics* 2020;**20**:e1800404
74. Suárez-Rivero J, Villanueva-Paz M, de la Cruz-Ojeda P, de la Mata M, Cotán D, Oropesa-Ávila M, de Lavera I, Álvarez-Córdoba M, Luzón-Hidalgo R, Sánchez-Alcázar J. Mitochondrial dynamics in mitochondrial diseases. *Diseases* 2016;**5**:1
75. Zemirli N, Morel E, Molino D. Mitochondrial dynamics in basal and stressful conditions. *Int J Mol Sci* 2018;**19**:564
76. Glancy B, Willis WT, Chess DJ, Balaban RS. Effect of calcium on the oxidative phosphorylation Cascade in skeletal muscle mitochondria. *Biochemistry* 2013;**52**:2793–809
77. Müller M, Ahumada-Castro U, Sanhueza M, Gonzalez-Billault C, Court FA, Cárdenas C. Mitochondria and calcium regulation as basis of neurodegeneration associated with aging. *Front Neurosci* 2018;**12**:1–8
78. Madreiter-Sokolowski CT, Waldeck-Weiermair M, Bourguignon M-P, Villeneuve N, Gottschalk B, Klec C, Stryeck S, Radulovic S, Parichatikanond W, Frank S, Madl T, Malli R, Graier WF. Enhanced inter-compartmental Ca 2+ flux modulates mitochondrial metabolism and apoptotic threshold during aging. *Redox Biol* 2018;**20**:458–66
79. Capel F, Demaison L, Maskouri F, Diot A, Buffiere C, Mirand PP, Mosoni L. Calcium overload increases oxidative stress in old rat gastrocnemius muscle. *J Physiol Pharmacol* 2005;**56**:369–80

(Received May 11, 2020, Accepted June 27, 2020)