Mitochondrial calcium: Transport and modulation of cellular processes in homeostasis and cancer (Review)

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Abstract. In addition to their role in providing cellular energy, mitochondria fulfill a key function in cellular calcium management. The present review provides an integrative view of cellular and mitochondrial calcium homeostasis, and discusses how calcium regulates mitochondrial dynamics and functionality, thus affecting various cellular processes. Calcium crosstalk exists in the domain created between the endoplasmic reticulum and mitochondria, which is known as the mitochondria-associated membrane (MAM), and controls cellular homeostasis. Calcium signaling participates in numerous biochemical and cellular processes, where calcium concentration, temporality and durability are part of a regulated, finely tuned interplay in non-transformed cells. In addition, cancer cells modify their MAMs, which consequently affects calcium homeostasis to support mesenchymal transformation, migration, invasiveness, metastasis and autophagy. Alterations in calcium homeostasis may also support resistance to apoptosis, which is a serious problem facing current chemotherapeutic treatments. Notably, mitochondrial dynamics are also affected by mitochondrial calcium concentration to promote cancer survival responses. Dysregulated levels of mitochondrial calcium, alongside other signals, promote mitoflash generation in tumor cells, and an increased frequency of mitoflashes may induce epithelial-to-mesenchymal transition. Therefore, cancer cells remodel their calcium balance through numerous mechanisms that support their survival and growth.

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1. Introduction

Calcium (Ca^{2+}) signaling regulates various physiological processes, including muscle contraction, neuron excitability, cellular secretion and cell migration [1,2]. At the cellular level, fine-tuned regulation of intracellular Ca^{2+} levels is essential for maintaining cell survival, cell function and mitochondrial dynamics, which are the focus of the first part of this review.

Total cellular Ca^{2+} levels are maintained through the sum of Ca^{2+} influx that occurs through the plasma membrane using cation channels and the release of Ca^{2+} from intracellular Ca^{2+} stores. The endoplasmic reticulum (ER) and mitochondria are the main Ca^{2+} stores. The role of mitochondria in calcium homeostasis is important because of its function as the main cellular chamber involved in ATP production, and because there is a link between mitochondrial levels of calcium and mitochondrial dynamics, function and metabolism. Mitochondria are also involved in cellular microdomains that regulate vital cellular processes [3,4].

Cancer remodels the systems that maintain cellular calcium homeostasis, including Ca^{2+} transporters and their regulators, Ca^{2+}-dependent enzyme activity and accessory molecules that will support tumor development. These processes are discussed in the second part of the present review.

2. Mitochondrial calcium homeostasis

Mitochondrial calcium homeostasis is regulated through a fine-tuned process that defines the capacity of the mitochondria

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for handling calcium. This process is critical for the regulation of aerobic metabolism and cell survival (5). In this manner, mitochondria an important role in modulating the amplitude and timing of intracellular Ca^{2+} signals through two mechanisms (6): Mitochondrial calcium buffering capacity and mitochondrial functional interactions with other channels or organelles.

Mitochondria possess a calcium buffering capacity that can handle levels of intracellular calcium between 50 and 500 nM in numerous types of normal cells (7-9). Through this buffering activity, mitochondria may dynamically manage transient alterations in calcium concentrations and may buffer severe Ca^{2+} overloads, which are often associated with pathological conditions (9). Notably, mitochondria can functionally interact with calcium-channel gating in the cell membrane of T lymphocytes. A prolonged elevation of cytosolic Ca^{2+} is required for T-cell activation, and functional mitochondria prevent a local accumulation of Ca^{2+} near sites that govern channel activation and maintain an opened Ca^{2+} release-activated Ca^{2+} (CRAC)-channel (10).

The close contact between mitochondria and cellular Ca^{2+} gates present in the ER and the cell membrane creates microdomains that allow the increase of mitochondrial calcium concentration ($[Ca^{2+}]_m$) in parallel to cytosolic Ca^{2+} signals, despite the low affinity of $[Ca^{2+}]_m$ uptake systems (11). Numerous close contacts have been observed between mitochondria and the ER, where, upon opening of the inositol 1,4,5-triphosphate (IP_3)-gated channels in the ER, the surface of the mitochondria is exposed to higher concentrations of Ca^{2+} than the bulk cytosol, which increases $[Ca^{2+}]_m$ (Fig. 1) (12,13). In addition, microdomains between the mitochondria and cell membrane allow the entrance of extracellular calcium through cell membrane channels to the mitochondria, providing a link between cellular activation and mitochondrial function (Fig. 1) (9). The rapid diffusion of intracellular Ca^{2+} avoids organelle overload (12).

3. Mitochondrial calcium transport

Calcium can be transported into the mitochondria using voltage-dependent anion channels (VDACs) that exist on the outer mitochondrial membrane (OMM) in excitable cells, including neurons, muscle cells and fibroblasts. VDACs can form a large conductance channel in lipid bilayers (14). Furthermore, VDACs exist in the cell membrane of neurons and retinal amacrine cell (15,16). VDACs can also transport other small molecules and ions; Ca^{2+} can regulate VDAC activity, thus controlling permeability to ions and small molecules through the OMM. Elevations of $[Ca^{2+}]$ to the micromolar range induce higher conductance and sustain VDAC opening. Notably, $[Ca^{2+}]_m$ uptake is often driven by spikes and oscillations in $[Ca^{2+}]$, which are characterized by short-lasting events; under physiological ionic conditions VDACs often present closed conformations (Fig. 1) (14).

Calcium is transported by the mitochondrial calcium uniporter (MCU), a ruthenium-red-sensitive uniporter that utilizes the negative potential across the inner mitochondrial membrane (IMM) (17). MCU pentamerizes in the IMM as part of a larger molecular weight complex; the other subunits are mitochondrial calcium uptake 1 and 2 (MICU1 and MICU2),

and the essential MCU regulator (EMRE). MICU1 and MICU2 serve as calcium sensors that allow Ca^{2+}-dependent opening of the MCU pore. EMRE is a single-pass membrane protein required to allow Ca^{2+} access (Fig. 1). When Ca^{2+} concentration around the MCU is >1 mM, the channel opens and allows Ca^{2+} to enter into the matrix that is driven by the large negative membrane potential of the IMM (18). In endothelial cells exposed to various glucose concentrations (2-30 mM), MCU and MICU1 are upregulated in a dose- and time-dependent manner. Therefore, endothelial cells under hyperglycemic stress exhibit cell dysfunction associated with $[Ca^{2+}]_m$ overload, which results in an increase in reactive oxygen species (ROS) that promotes apoptosis (19).

The primary mechanism underlying calcium extrusion in excitable cells is the $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX); this transporter exchanges mitochondrial matrix calcium for external sodium or lithium, hence its name (20,21). Therefore, to allow activity of the Na^+/Ca^{2+} antiporter, matrix Na^+ must be exchanged for external H^+ by a Na^+/H^+ exchanger (22). In addition, Ca^{2+} efflux might occur via a Ca^{2+}/H^+ exchanger, which is expressed in acidic organelles, including mitochondria (22,23). The mitochondrial K^+/H^+ exchanger (KHE) also participates in mitochondrial calcium dynamics; however, KHE discriminates poorly between K^+ and other monovalent cations, extruding Na^+ by H^+. Another IMM protein that has K^+/H^+ exchange activity is the leucine zipper-EF-hand containing transmembrane protein 1 (LETM1). Lowering LETM1 expression via short hairpin RNA hampers mitochondrial K^+ or Na^+/H^+ exchange. Furthermore, LETM1 downregulation decreases Na^+/Ca^{2+} exchange mediated by the NCLX (22). Furthermore, LETM1 is able to transport Ca^{2+} in a pH-dependent manner. When the pH shifts from acidic to alkaline, LETM1 opens a central cavity allowing Ca^{2+}/H^+ antiporter (24). Therefore, a fine-tuning interplay exists among $[Ca^{2+}]$, $[Na^+]$, $[Li^+]$, $[K^+]$ and $[H^+]$ at the mitochondrial matrix and in the intermembrane space where calcium is a key player (Fig. 1).

4. Calcium interplay between ER and mitochondria shapes mitochondrial function and dynamics

Mitochondria associated membrane (MAM) is a key microdomain in calcium homeostasis. At the cellular level, calcium has a role in how mitochondria interact with other organelles, including the ER, which can also affect mitochondrial function and dynamics. The spatially restricted domain created between the ER and the mitochondria is known as the MAM. Notably, ~20% of the mitochondria surface is in close contact with the ER, where the ER protein vesicle-associated membrane protein associated protein B (VAPB) is the scaffold that tethers the ER to the mitochondrial protein, protein tyrosine phosphatase interacting protein 51 (Fig. 1) (13). Another molecule that participates in tethering the ER and mitochondria together is mitofusin 2 (MFN2), which also has GTPase activity (25), wherein B-cell lymphoma-2 (Bcl-2) related protein ovarian killer increases MFN2 to cause tethering of the ER and mitochondria to facilitate mitochondrial fission (26). Additionally, MAM functions as a critical membrane contact site for lipid synthesis and exchange (26). The importance of MAM communication is underlined in various human diseases, including cancer (13,26-29).
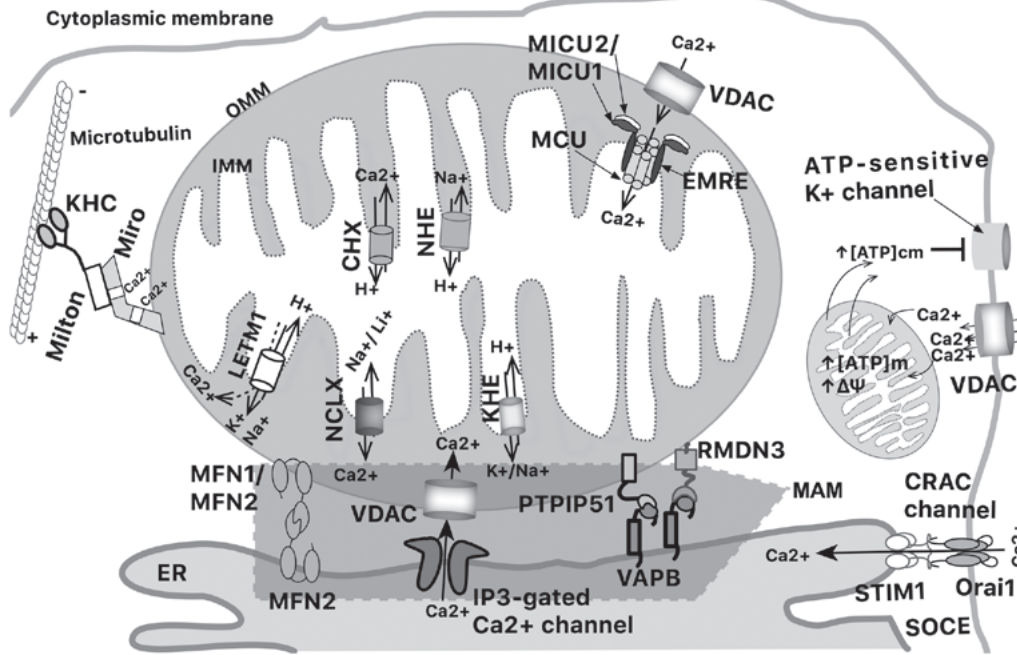
Figure 1. Schematic representation of mitochondrial calcium interplay. Calcium can be directly transported from the cytoplasmic membrane into mitochondria or ER using VDAC and CRAC channels. VDAC/MCU and STIM1 introduce calcium into mitochondria and ER, respectively. IP$_3$Rs export calcium from ER to mitochondria at MAMs where various pairs of molecules support the union between mitochondria and ER. A fine-tuning interplay exists among different cations that participate in mitochondrial calcium extrusion. Dotted arrow represents pH-dependent transport of Ca^{2+}. CHX, Ca^{2+}/H^+ exchanger; CRAC, calcium release-activated channels; EMRE, essential MCU regulator; ER, endoplasmic reticulum; IP$_3$R, inositol 1,4,5-trisphosphate receptor; KHC, kinesin heavy chain; KHE, mitochondrial K^+/H^+ exchanger; LETM1, leucine zipper-EF-hand containing transmembrane protein 1; MAM, mitochondria-associated membrane; MCU, mitochondrial calcium uniporter; MICU1/2, mitochondrial calcium uptake 1 and 2; NCLX, Na^+/Ca^{2+} exchange; NHE, Na^+/H^+ exchanger; PTPIP51, protein tyrosine phosphatase interacting protein 51; RMDN3, regulator of microtubule dynamics 3; SOCE, store-operated Ca^{2+} entry channels; STIM1, stromal interaction molecule 1; VAPB, vesicle-associated membrane protein associated protein B; VDAC, voltage-dependent anion channels.

Notably, the ER supplies Ca^{2+} directly to mitochondria via the MAM through the use of IP$_3$ receptors (IP$_3$Rs) (13). IP$_3$Rs are tetrameric ER-resident Ca^{2+} channels that release Ca^{2+} from the ER into the cytosol in response to IP$_3$ (Fig. 1) (30). Three isoforms comprise the IP$_3$R family and the majority of cell types express two or even all three isoforms. For example, secretory granules of human astrocytes can contain all three IP$_3$R isoforms (31). Although, IP$_3$R-1 is the most widely expressed and appears to be ubiquitous in animal tissues, it can be highly expressed in cells from the mouse central nervous system (32). IP$_3$R-2 and -3 are highly expressed in human hematopoietic and lymphatic cells (33). In this regard, IP$_3$Rs seem to be essential for constitutive Ca^{2+} release from the ER to mitochondria, in order to maintain sufficient mitochondrial NADH production to support mitochondrial bioenergetics. In DT40-KO cells, which is a chicken B cell line in which the three IP$_3$R isoforms are genetically deleted, the absence of Ca^{2+} transfer inhibits pyruvate dehydrogenase and activates 5' AMP-activated protein kinase (AMPK), which activates pro-survival autophagy via a mammalian target of rapamycin (mTOR)-independent mechanism. AMPK phosphorylates substrates to limit anabolic pathways that consume ATP and to initiate catabolic pathways that support oxidative phosphorylation (OXPHOS) (34).

Role of Ca^{2+} in mitochondrial function
Increasing evidence has revealed how calcium regulates mitochondrial function. Notably, high glucose levels promote a sustained increase in $[Ca^{2+}]_m$, cytoplasmic calcium concentration ($[Ca^{2+}]_c$) and insulin secretion; these effects can be abolished by inhibitors of voltage-gated Ca^{2+} channels in INS-1 pancreatic β-cells. The increase in calcium levels promotes the synthesis of mitochondrial ATP, accompanied by increased levels of NADH and NADPH cofactors (35). Calcium-associated activation of mitochondrial dehydrogenases, including pyruvate and 2-oxoglutarate dehydrogenase complexes, NAD$^+$-isocitrate dehydrogenase and inorganic pyrophosphatase, may sustain the reduction of NAD$^+$ and NADP$^+$ cofactors. All these enzymes are highly sensitive to alterations in calcium concentration inside the mitochondrial matrix (9,11,35,36). For example, $[Ca^{2+}]_m$ <1 mM is required to induce the full activation of pyruvate dehydrogenase (PDH)-phosphate phosphatase, which removes the phosphate group from PDH-phosphate, and increases the proportion of active and dephosphorylated PDH. Two other examples include 2-oxoglutarate dehydrogenase that catalyzes the irreversible reaction of the oxidative decarboxylation of 2-oxoglutarate and NAD$^+$-isocitrate dehydrogenase that responds to changes in $[Ca^{2+}]_m$ varying between 1 and 20 mM (37).

Notably, in single islet β-cells, extracellular glucose concentrations (3-30 mM) increase $[ATP]_m$ in the mitochondria located close to the cytoplasmic membrane (cm). This phenomenon leads to increased $[ATP]_{cm}$, which is essential for sustained closure of the ATP-sensitive K^+ channels that additionally causes Ca^{2+} influx into the cell. The mitochondria located close to the cytoplasmic membrane also have access to a privileged elevation of $[Ca^{2+}]_{cm}$. This calcium enters

mitochondria, progressively activating Ca^{2+}-dependent mitochondrial enzymes to increase $[ATP]_m$ that establishes a feed-forward process (Fig. 1) (36).

Mitochondrial matrix calcium can also activate ATP synthesis and adenylate transport (38). The increase in [ATP] depends on the amplitude of Ca^{2+} elevations in the mitochondrial matrix and the availability of mitochondrial substrates. Notably, a Ca^{2+} increase induces a long-lasting effect that persists even after the elevation has finished, which denotes a cellular memory that allows prolonged metabolic activation in stimulated cells (11).

Activation of ATP synthesis by $[Ca^{2+}]_m$ is concomitantly accompanied by a rise in mitochondrial membrane potential, which is the driving force for Ca^{2+} accumulation (36,37). This has been shown in cerebellar granule neurons, where *in vitro* neuronal stimulation induced by mitochondrial depolarization with high concentrations of KCl results in the entry of cytosolic Ca^{2+} into the mitochondria (3). Conversely, a decrease in mitochondrial membrane potential inhibits $[Ca^{2+}]_m$ uptake (3).

In HL-1 cardiomyocytes, sustained elevation of intracellular $[Ca^{2+}]$ can cause hypertrophy. During such Ca^{2+} elevations, mitochondria accumulate calcium rapidly, which induces the opening of permeability transition pores (PTP), matrix swelling, OMM rupture and cell death (39). High concentrations of calcium (300 mM) decrease ATP production >2-fold in cardiomyocytic mitochondria; however, incubation of these mitochondria with Ca^{2+} and muscle fructose 1,6-bisphosphatase (FBP) allows recovery of ATP levels, thus suggesting that FBP blocks the inhibitory effects of high $[Ca^{2+}]$ (39). Muscle FBP has two predominant oligomeric states that determine its subcellular localization. Only dimeric FBP interacts with mitochondria and protects them against stress stimuli (40). Therefore, as a survival mechanism associated with the elevation of calcium, cardiomyocytes promote the binding of FBP to mitochondria. FBP, in addition to being a regulatory enzyme of glyconeogenesis, can also join to mitochondria preventing their dysfunction by calcium stress. FBP interacts with mitochondrial proteins involved in regulating membrane permeability by reducing Ca^{2+}-induced PTP opening; FBP also interacts with mitochondrial proteins involved in energy homeostasis, which causes stimulation of ATP synthesis (39).

Role of Ca^{2+} in mitochondrial dynamics
Ca^{2+} influx into mitochondria has important consequences on the organelles themselves. For example, Ca^{2+} influx through VDAC and MCU induces rapid mitochondrial fission by phosphorylation at serine 600 of the dynamin-related protein 1 (DRP1) in neurons (41). DRP1 also has GTPase activity and serves a critical role in mitochondrial fission (42). In addition, the increase in $[Ca^{2+}]_c$ promotes the translocation of DRP1 to mitochondria in cardiomyocytes and in cadmium-treated human and rat liver cells (43,44). Mitochondrial fission events have been located at the MAM compartment (26), where Ca^{2+} is a critical regulator of mitochondrial morphology since mitochondrial Ca^{2+} overload leads to mitochondrial fragmentation, whereas mitochondrial Ca^{2+} depletion results in mitochondrial hyperfusion (45).

Mitochondria depend on microtubules for their intracellular motility via a Ca^{2+}-regulated mechanism. This movement capacity of mitochondria allows appropriate distribution of these organelles for local ATP supply and Ca^{2+} buffering. Using this movement, mitochondria is settled in perinuclear clusters or in clusters near the cytoplasmic membrane. In this regard, the KHC/Milton/Miro complex regulates Ca^{2+}-dependent mitochondrial motility. The Miro protein joins to the OMM. Miro contains a pair of EF-hand Ca^{2+}-binding motifs that will permit interaction with the motor domain of kinesin-1 through the Milton adaptor protein (Fig. 1) (46). Calcium-dependent arrest of mitochondrial motility is required to position mitochondria where the cell needs Ca^{2+}-buffering or an increased ATP supply, as has been observed in neurons and other cells (46,47). For example, motility of astrocytic mitochondria is inversely related to intracellular calcium concentrations ($[Ca^{2+}]_i$); elevated $[Ca^{2+}]_i$ immobilizes astrocytic mitochondria, whereas low $[Ca^{2+}]_i$ under resting conditions causes mitochondria to be highly mobile (47). The mitochondrial fission-fusion machinery is also associated with the mitochondrial motility machinery.

Ca^{2+} participation in mitochondrial dynamics also includes mitophagy, which is a highly specialized process that removes dysfunctional mitochondria. This has been observed in neurodegenerative diseases, and calcium dysregulation and ROS production are potential mitophagy inducers (48,49). Mitophagy protects cells from damage that could occur from a disordered mitochondrial metabolism. Phosphatase and tensin homolog deleted on chromosome 10-induced kinase 1 (PINK1) is a mitochondrial protein that preserves mitochondrial integrity, and also participates in mitophagy activation. Upon mitochondrial depolarization, PINK1 and beclin 1 (BECN1) selectively relocalize to the surface of the damaged mitochondria, particularly at the MAM contact. This anchoring of PINK1 and BECN1 recruits proteins, such as ubiquitin and PARK2/Parkin, giving rise to autophagosome formation, thus resulting in mitophagy of damaged mitochondria (50). PINK1 localized at MAM also modulates mitochondrial calcium levels (48,50).

5. Calcium signals control cell migration and apoptosis

In migrating eukaryotic cells, calcium signals control cell migration through the regulation of forward movement and cell adhesion (2). For example, Ca^{2+}-influx channels regulate trailing edge contraction in the migration of growth factor-induced fibroblast cells and spontaneously polarized macrophages (1,51). In particular, inhibition of the extracellular Ca^{2+} influx in RAW macrophages induces the loss of phosphoinositide 3-kinase (PI3K) activity at the leading edge, disassembly of filamentous actin (F-actin) and cessation of ruffling. Ca^{2+}-sensitive enzyme protein kinase Ca (PKCa), which is an actin regulatory protein, is enriched at the leading edge of macrophages. This PKCa enrichment is affected by blockade of Ca^{2+} influx, inhibition of PI3K activity or F-actin depolymerization (51). PKCa can synergistically regulate migration by phosphorylating myosin, by regulating the cytoskeleton or by turning over integrin complexes (2).

Calcium signals, in addition to regulating forward movement, can also control cell adhesion. Pulsatile front retraction and cell adhesion of human umbilical vein endothelial cells are locally supported by Ca^{2+} pulses near the leading edge caused by ER Ca^{2+} depletion and stromal interaction molecule 1 (STIM1) activation. STIM1 likely acts locally on adhesion by enhancing Ca^{2+} influx and reloading

Table I. Tumor suppressor proteins and oncoproteins that localize to the mitochondria-associated membrane.

A, Tumor suppressor proteins

First author, year	Name	Reference
Raturi et al, 2016	Thioredoxin-related transmembrane protein	(101)
Nutt et al, 2002; Scorrano et al, 2003	Bcl-2-associated X, apoptosis regulator	(102,103)
Nutt et al, 2002; Scorrano et al, 2003	Bcl-2 antagonist/killer 1	(102,103)
Echeverry et al, 2013	Bcl-2 related protein ovarian killer	(104)
Giorgi et al, 2010	Promyelocytic leukemia protein	(105)
Bononi et al, 2013	Phosphatase and tensin homolog deleted on chromosome 10	(106)
Verfaillie et al, 2012	RNA-dependent protein kinase-like ER kinase	(107)
Li et al, 2008	ER-associated apoptosis-involved protein	(108)
Seervi et al, 2013	ER oxidoreductin-1α	(58)
Iwasawa et al, 2011	Fission, mitochondrial 1	(109)
Iwasawa et al, 2011	B cell receptor-associated protein 31	(109)
Namba et al, 2013	Cell death inducing p53 target 1	(110)

B, Anti-apoptotic molecules

	Name	Reference
Betz et al, 2013	Mammalian target of rapamycin complex 2	(55)
Monaco et al, 2015	Bcl-2	(62)

Bcl-2, B-cell lymphoma 2; ER, endoplasmic reticulum.

ER Ca^{2+} stores in the front of the cell to permit local Ca^{2+} pulses (Fig. 1) (2). Cancer cell migration will be described below.

Ca^{2+} transfer between the ER and mitochondria represents a critical signal in the induction of apoptosis (28). One of the best-characterized mechanisms is via the p53 pathway. The p53 protein is a master tumor-suppressor protein that promotes apoptosis through two mechanisms: As a transcription factor, p53 controls cell-death programs within the nucleus (52), or through a Ca^{2+}-dependent mechanism modeling MAM cross talk. The p53 protein interacts with sarco/ER Ca^{2+}-ATPase (SERCA) pumps causing an alteration in their oxidative state. This leads to an increased Ca^{2+} load that is transferred directly to the mitochondria that causes changes in mitochondrial morphology and induction of apoptosis (28). Depolarization of the mitochondria triggered by high Ca^{2+} levels is followed by a decrease in ATP synthesis, leading to cell death (39). Morphological alterations in mitochondria caused by Ca^{2+} overload result in the opening of PTPs, mitochondrial fragmentation and cytochrome c release, which are all factors that lead to apoptotic cell death (28).

6. Calcium interplay is remodeled by cancer: Alterations in MAM and mitochondrial dynamics

Cancer alters the architecture of MAM to support survival and aggressiveness
The present review discussed the mechanisms where the role of Ca^{2+} is essential for sustaining cellular homeostasis.

However, in cancer, increasing evidence has revealed a link between alterations in mitochondrial Ca^{2+} homeostasis and malignant phenotypes, including cell survival, invasiveness, migration, apoptosis evasion and drug resistance (53,54).

Notably, MAM domains are involved in various important processes in tumor cells that allow their survival, including adhesion, motility, invasion, metastasis, autophagy and apoptosis (28,55,56). Cancer cells remodel MAM architecture by modifying the expression levels of ER and mitochondrial Ca^{2+} channel proteins, their associated binding partners, or their regulators (53). For example, in HeLa cells, a detailed microscopy study demonstrated that a fraction of the mitochondrial pool is in close contact with the ER cisternae in these highly localized MAM domains (12). These contacts are also increased between mitochondria and ER in prostate cancer cells that have acquired cancer stem-like properties (57).

Numerous tumor suppressor proteins have been demonstrated to be localized to the ER and MAMs where they regulate cell death (Table I). Some of these suppressor proteins directly affect calcium flux; for example, silencing of ER oxidoreductin-1α significantly inhibits calcium release from the ER, inducing cell death (58). A reduction in the Bcl-2/Bcl-2-associated X, apoptosis regulator ratio also leads to ER Ca^{2+} depletion, which induces cytosolic Ca^{2+} overload and causes the loss of mitochondrial membrane potential (59).

IP_3R-3 is an oncoprotein differentially expressed in colorectal carcinoma that is absent in normal colorectal mucosa (Fig. 2). Increased expression of IP_3R-3 is strongly correlated with metastasis and decreased 5-year survival (60).

In addition, IP$_3$R-3, but not the type 1 or 2 isoforms, is overexpressed in gastric cancer cell lines established from malignant ascites (61). Conversely, IP$_3$R-3 is involved in mediating pro-apoptotic Ca^{2+} signals from the ER to mitochondria in HeLa cells (62). Although IP$_3$R-3 colocalizes more closely with mitochondria more efficiently transmitting Ca^{2+} signals, in colorectal cancer, IP$_3$R-3 expression inhibits rather than promotes apoptosis, possibly by participating in a localized signaling domain mechanism that remains to be clarified (60).

Some anti-apoptotic molecules are also localized to the MAM and modulate cell death (Table I). mTOR complex 2 (mTORC2) controls MAM integrity and mitochondrial function via protein kinase B (AKT)-mediated phosphorylation of IP$_3$R and hexokinase 2 (55). In non-small cell lung carcinoma (NSCLC) cells resistant to erlotinib, a tyrosine kinase inhibitor (TKI), epidermal growth factor receptor (EGFR)-TKI resistance is reduced in response to rituximab, an anti-cluster of differentiation 20 monoclonal antibody. Briefly, NSCLC cells that carry the EGFR T790M-mutation are resistant to erlotinib treatment; however, the addition of rituximab significantly inhibits proliferation and cell survival. The proposed mechanism involves a reduction in the phosphorylation of EGFR, mTOR and mTORC2, which is associated with inhibition of mTORC2 localization to MAM; consequently, cell growth is inhibited (63).

Bcl-2 and Bcl-extra large (Bcl-XL) proteins exert part of their anti-apoptotic functions by directly binding to ER-localized IP$_3$Rs and to OMM-localized VDAC1, respectively. The joining of Bcl-2 and Bcl-XL to these Ca^{2+} transport systems will negatively affect Ca^{2+} transfer and apoptosis (Fig. 2) (62).

Calcium levels affect mitochondrial dynamics
To satisfy cellular demands, mitochondria continually change their shape and function; therefore, the combined actions of fusion and fission determine the balance between oxidative and glycolytic metabolisms. In cancer, the ability of tumor cells to survive has been associated with unbalanced mitochondrial dynamics, as it has been demonstrated in lung, pancreatic, breast and oncocytic thyroid cancers, among others (64-67). Notably, feedback regulation has been established between calcium signaling and mitochondrial dynamics to coordinate regulation of numerous tumor cellular processes (68). For example, in melanoma cells treated with three compounds, a mitochondrial Na$^+$/Ca^{2+} exchanger inhibitor (CGP-37157) and two OXPHOS inhibitors (antimycin A and FCCP), a fast and persistent rise in [Ca^{2+}]$_m$ is detected. These compounds increase tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) sensitivity, switching from apoptosis to nonapoptotic cell death. The [Ca^{2+}]$_m$ overload increases mitochondrial fragmentation, whereas [Ca^{2+}]$_m$ removal induces mitochondrial hyperfusion. Notably, both actions affecting mitochondrial dynamics exacerbate TRAIL-induced mitochondrial rearrangements and cytotoxicity. This study indicated that an appropriate level of mitochondrial calcium is essential for maintaining mitochondrial dynamics associated with tumor cell survival (45).

In liver cancer cells, increased [Ca^{2+}]$_c$ promotes mitochondrial fission by upregulating DRP1 and fission, mitochondrial 1

genes via nuclear factor of activated T cells 2 (NFATC2) and c-Myc activation, respectively. NFATC2 is a calcium responsive transcription factor that it is overactivated in several types of cancers (68).

MFN2 is a protein that promotes mitochondrial fusion. This molecule is downregulated in patients with liver cancer and low levels of MFN2 are associated with a poorer prognosis. Notably, MFN2 expression is lower in tumor tissues than in adjacent noncancer tissues. *In vitro* upregulation of MFN2 in the liver cancer cell line HepG2 triggers Ca^{2+} influx from the ER into mitochondria, increasing [Ca^{2+}]$_m$, ROS production and reducing mitochondrial membrane potential; all these phenomena lead to tumor cell apoptosis. These findings highlight the role of MFN2 as a tumor suppressor protein (69). Furthermore, it has been described that microRNA (miR)-761 binds the 3'-untranslated region of MFN2. The upregulation of miR-761 is associated with MFN2 downregulation; therefore, it may support cell proliferation, cell migration and invasiveness of liver tumors. Inhibition of miR-761 affects mitochondrial function and inhibits cell proliferation, migration and invasion of liver cancer *in vivo* and *in vitro* (Fig. 2) (70).

7. Mitochondrial calcium and mitoflashes in epithelial-to-mesenchymal transition

Ca^{2+} signals regulate proliferation in a spatial- and temporal-dependent manner. Whereas nuclear Ca^{2+} signals may regulate cell proliferation, the increase of [Ca^{2+}]$_m$ may regulate apoptosis in epithelial cells (62,71). Nevertheless, calcium signaling may support loss of the balance between cell proliferation and apoptosis, favoring cellular proliferation and inhibiting apoptosis in cancer. Enhanced cytosolic Ca^{2+} signaling has been suggested to contribute to the transition from normal colonic epithelial cells to adenoma, which finally transforms to carcinoma (60). The epithelial-to-mesenchymal transition (EMT) is a critical process during progression of tumor metastasis (72). The loss of cell-cell adhesion is promoted by the transformation of polarized epithelial cells into highly motile mesenchymal cells (73). In MCF-7 breast cancer cells, inhibition of the Oct4 transcription factor or treatment with transforming growth factor β1 (TGFβ1) promotes migration and invasion. This EMT induction is accompanied by upregulation of STIM1 and ORAI calcium release-activated calcium modulator 1 (Orai1), two major components of store-operated Ca^{2+} entry channels (SOCE). STIM1 serves as a Ca^{2+}-sensor, and Orai1 is an essential pore-forming component of SOCE channel (Fig. 2) (74). Blockage of Ca^{2+} influx by silencing STIM1 partially rescues the EMT initiated by Oct4 downregulation, indicating that other calcium channels possibly take part in increased calcium influx (72). The SOCE pathway is activated by the release of Ca^{2+} from the ER that causes the oligomerization of STIM1. STIM1 binds the plasma membrane Ca^{2+} channel Orai, which causes it to open (75). Nevertheless, this mechanism requires functional mitochondria and the inhibition of MCU slowly deactivates SOCE (6).

In addition to TGFβ, EGF is associated with EMT induction in the breast cancer cell line MDA-MB-468, through upregulating the mRNA expression levels of the ATP-binding cassette C3 (ABCC3). The EGF-induced transcriptional upregulation of ABCC3 is dependent on calcium signals.
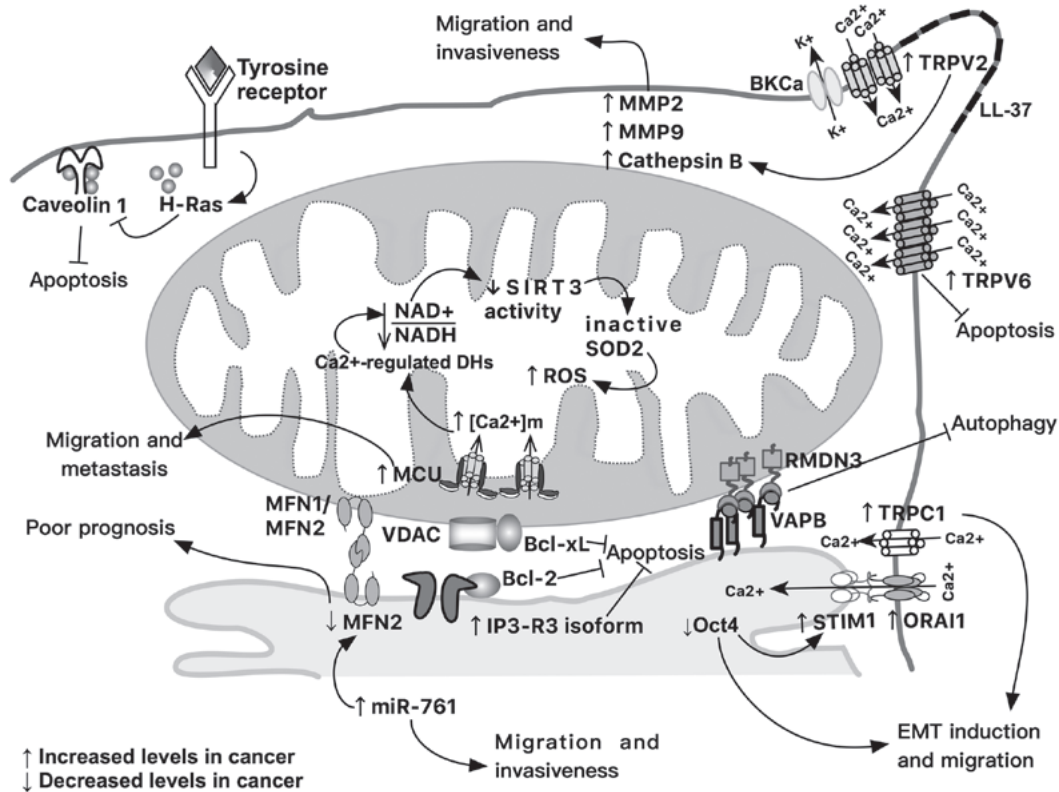
Figure 2. Tumor modifications in mitochondrial calcium interplay that support invasiveness, migration, apoptosis evasion and induction of epithelial-to-mesenchymal transition. Note that not all these modifications occur at the same time and in the same tumor. MMP, matrix metalloproteinase; BKCa, large-conductance Ca^{2+}-activated K^+ channel; DH, dehydrogenase; EMT, epithelial-to-mesenchymal transition; Oct4, octamer-binding transcription factor 4; SIRT3, sirtuin 3; SOD2, superoxide dismutase 2; TRPC1, transient receptor potential cation channel C1; TRPV, transient receptor potential cation channel subfamily vanilloid.

This calcium-dependent regulation of ABCC3 occurs via the calcium permeable ion channel transient receptor potential cation channel C1 (TRPC1) (76). Furthermore, a previous study suggested that the TRPC1 channel contributes to Orai1-mediated Ca^{2+} influx in the EMT of MDA-MB-468 cells (Fig. 2) (77). Additionally, TRPC1-mediated calcium influx has been linked to EGF-stimulated EGFR activation in non-small cell lung cancer cells by promoting cellular proliferation (78).

Mitochondrial calcium levels also regulate certain discrete and stochastic mitochondrial events called mitoflashes, which comprise a burst of superoxide production accompanied by transient depolarization and alkalization in the mitochondrial matrix (79). Transient opening of the mitochondrial PTP (mPTP) seems to precede the mitoflash. Mitoflash biogenesis can be activated by $[Ca^{2+}]_m$ increase, ROS and mPTP promoters, but suppressed by ROS scavengers and mPTP inhibitors (80). Hyperosmotic or oxidative stress and ROS-dependent apoptosis are associated with an increased frequency of mitoflashes, which arises as a bioindicator of cellular dysfunction (81). The frequency of mitoflashes has been reported to transiently increase during the early stages of somatic cell reprogramming, establishing a link between the role of mitoflashes and the acquisition of pluripotency (82). Other factors that may modulate mitoflash occurrence rate are aging and cancer (80).

Proteins such as EF-hand domain family member D (EFHD), solute carrier family 25 member 25 (SLC25A25), solute carrier family 25 member 23 and MICU1 are considered mitoflash activators. Notably, all of them contain Ca^{2+}-binding motifs. In addition, LETM1 is considered a mitoflash inhibitor that responds to hyperosmotic stress (Fig. 3). Recently, optic atrophy 1 protein (OPA1) has emerged as a regulator of the occurrence of mitoflashes, which is an independent process from fusion; OPA1 stabilizes respiratory chain supercomplexes in a conformation that enables mitochondria to compensate a fall in mitochondrial membrane potential by an explosive-pH flash (Fig. 3) (81).

EFHD1 has been involved in the alteration of mitochondrial function to control cell survival or neural differentiation. EFHD1 is also known as mitocalcin and has Ca^{2+} binding characteristics; this molecule acts as a Ca^{2+} sensor enhancing mitoflash responses (79). Another example is SLC25A25, which functions as a Ca^{2+}-regulated shuttle of ATP-Mg^{2+} and pyrophosphate across the IMM. SLC25A25 has been reported to be upregulated by 3.2-fold in the RC77T/E human prostate tumor cell line (83). In this sense, the expression of the long noncoding RNA relating to SLC25A25 (SLC25A25-AS1), which inhibits SLC25A25 translation, is significantly decreased in tumor tissues and serum of patients with colorectal cancer. *In vitro* overexpression of SLC25A25-AS1 significantly inhibits proliferation and colony formation in colorectal cancer cell lines; in addition, downregulation of SLC25A25-AS1 enhances chemoresistance and promotes the EMT process (84).

8. Calcium: A critical regulator of tumor migration, invasiveness and metastasis

Ca^{2+} signals regulate polarization, speed and the direction of movement of migrating cells. Cancer cells migrate by applying
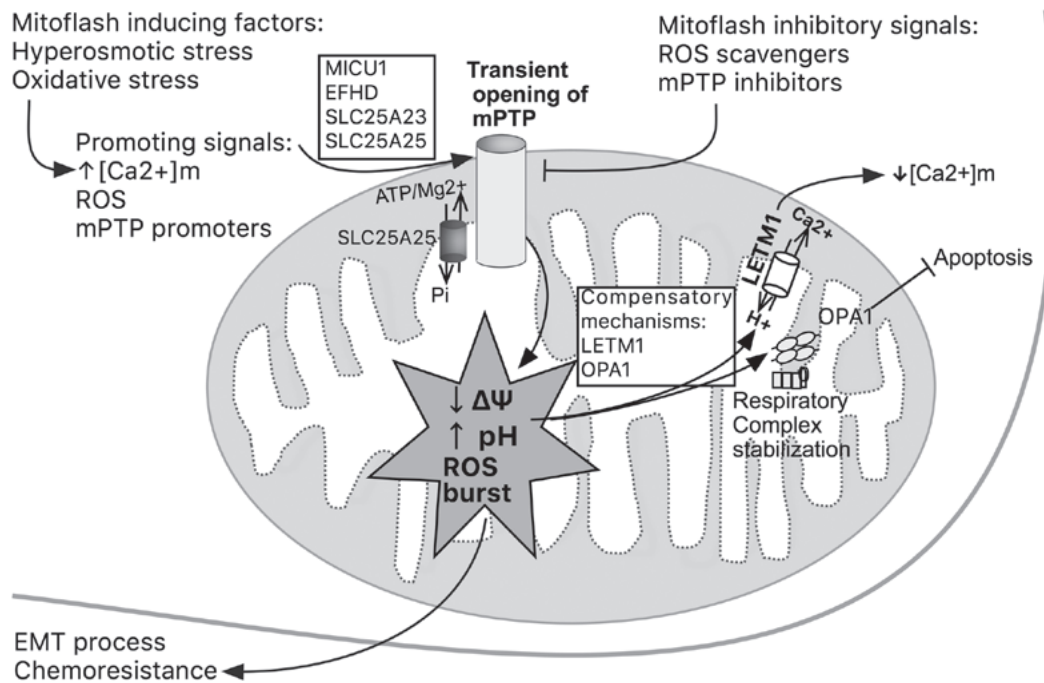
Figure 3. Schematic diagram of mitoflash biogenesis. Inducing factors promote signals that may act through various proteins. These proteins have a Ca^{2+}-binding site and participate in the transient opening of mPTP just before mitoflash occurrence. Subsequently, LETM1 and OPA1 proteins conform different compensatory mechanisms to avoid apoptosis or to diminish $[Ca^{2+}]_m$. An increased frequency of mitoflashes has been associated with EMT and chemoresistance. $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; EFHD, EF-hand domain family member D; EMT, epithelial-to-mesenchymal transition; mPTP, mitochondrial permeability transition pore; OPA1, optic atrophy 1 protein; SLC25A25, solute carrier family 25 member 25.

rearward forces against extracellular media. Freely migrating cancer cells move by inducing the formation of lamellipodia that are the extension of leading edge protrusions (85). Contractility-driven bleb formation has recently emerged as another mechanism of motility in mesenchymal precursors (86). Two sublines of WC256 carcinosarcoma cells (LC and BC) were transformed to observe mesenchymal migration and ameboid migration, and to find differences in electrotactic motility. LC and BC WC256 cells can efficiently drive electrotactic migration by forming lamellipodia and membrane blebs, respectively (87). Lamellipodia attach to the substratum, and contraction of the trailing rear edge moves the cell toward the lamellipodia. Blebs, on the other hand, are cellular protrusions expanded by hydrostatic pressure generated by a contractile actomyosin cortex. Bleb location appears to be directly controlled by the Rho pathway and by alterations in intracellular Ca^{2+} concentrations (87). Membrane rigidity is increased at the leading edge of pseudopodia and retracting tail in breast cancer cell lines by locally binding LL-37 to cytoplasmic membrane. LL-37 is a peptide released from the C-terminus of the human cathelicidin antimicrobial protein hCAPT18. LL-37 membrane binding activates PI3K/AKT signaling. Subsequently, AKT induces the recruitment of the transient receptor potential cation channel subfamily vanilloid member 2 (TRPV2) transporter from intracellular vesicles to plasma membrane of pseudopodia (Fig. 2). TRPV2 increases calcium that is accompanied by K^+ efflux through the BKCa channel (88). These local Ca^{2+} signals govern forward movement by regulating lamellipodia retraction and strengthen local adhesion to the extracellular matrix. Each Ca^{2+} pulse triggers contraction of actin filaments that activates myosin light-chain kinase and myosin II behind the leading edge (85). Activation

of Ca^{2+}-dependent kinases can also indirectly influence actin dynamics (51).

MCU upregulation significantly increases migration *in vitro* in MCF-7 breast cancer cells. Consistently, MCU protein levels are increased in biopsy samples from breast cancer patients who present with metastasis (Fig. 2) (89). Blocking Ca^{2+} influx using Ni^{2+} inhibits migration of mouse 4T1 and human MDA-MB-231 breast tumor cells, highlighting a link between Ca^{2+} influx and migration (1).

Metastasis is a multistep process where cancer cells from a primary tumor site spread to distant organs and form new tumors, and calcium is a critical regulator of this process (74,90). Using matrix metalloproteinases (MMPs) and cathepsins, invasive cells degrade surrounding tissue, and Ca^{2+} influx can affect the activity of these enzymes. In human prostate cancer, the development and progression of cancer to an aggressive castration-resistant phenotype are characterized by *de novo* expression of the TRPV2 channel. The role of TRPV2 is to maintain elevated cytosolic Ca^{2+} and to induce expression of MMP2, MMP9 and cathepsin B, enhancing a cell migration-invasive phenotype (Fig. 2) (91).

The metastatic capacity of tumor cells is also enhanced by upregulation of MCU expression. MCU increases mitochondrial Ca^{2+} uptake, which promotes ROS production, favoring a metabolic shift from oxidative to glycolytic metabolism. For example, MCU overexpression promotes MMP2 expression and cell motility of liver cancer metastasis in a nude mouse model, where intrahepatic and distal lung metastasis are promoted through a ROS-activated c-Jun N-terminal kinase pathway (54). Briefly, mitochondrial Ca^{2+} elevation increases the activity of Ca^{2+}-sensitive dehydrogenases, which diminishes the $NAD^+/NADH$ ratio. The NAD^+-dependent

deacetylase activity of sirtuin 3 (SIRT3) deacetylates superoxide dismutase 2 (SOD2), and the deacetylated and active form of SOD2 decreases ROS levels. However, low levels of NAD$^+$, caused by Ca^{2+}-sensitive dehydrogenases, inhibit SIRT3 activity, and the inactive form of SOD2 allows accumulation of ROS, which activates the c-Jun N-terminal kinase pathway (Fig. 2) (54). Furthermore, [Ca^{2+}]$_m$ levels are also increased in HepG2 cells that are exposed to high glucose levels (33 mM). Nevertheless, when MCU is inhibited, high glucose levels induce decreased generation of ROS and protection against inflammatory effects (92). Silencing MCU in cultured HeLa cells severely abrogates mitochondrial Ca^{2+} uptake, whereas mitochondrial respiration and membrane potential remain fully intact (17). Under these conditions, an oxidative metabolism might be favored and cell motility could be diminished.

The suppression of Ca^{2+} influx inhibits the AKT pathway in breast cancer cells (4T1, TUBO-P2J and HCC70 cell lines), thus resulting in the prevention of migration (90). Higher MCU expression has also been detected in MDA-MB-231 cells compared with in MCF-7 cells (89). MDA-MB-231 cells, which are more invasive than other breast cancer cells (4), have an elevated [Ca^{2+}]$_m$ uptake that is essential for the metabolic shift from oxidative to glycolytic metabolism that occurs during cancer metastasis (89). miR-340 can target and inhibit MCU, which consequently decreases [Ca^{2+}]$_m$ accumulation, metastatic cell motility and the matrix invasiveness of breast cancer cells (89). Additionally, STIM1 and Orai1 inhibition reduces serum-induced *in vitro* cell migration and *in vivo* metastasis formation of MDA-MB-231 breast cancer cells (74). Molecular components of the SOCE also regulate breast cancer metastasis by replenishing depleted ER Ca^{2+} reserves. Inhibition of SERCA with cyclopiazonic acid or by agonist stimulation with ATP (77), or signaling mechanisms such as activated G-protein-coupled or tyrosine kinase receptors (4), may deplete ER Ca^{2+} stores. As further proof, inhibition of MCU by ruthenium red or specific small interfering (si)RNAs induces mitochondrial depolarization in MDA-MB-231 cells, which inhibits SOCE through CRAC channels and abrogates cancer cell migration (4).

9. Mitochondrial calcium in tumor apoptotic failure and tumor autophagy

Tumor cells have developed mechanisms where alterations in mitochondrial metabolism inhibit the intrinsic apoptotic pathway. In this context, the oncoprotein Ras promotes the failure of mitochondrial-dependent apoptosis, which induces neoplasia via an interaction with caveolin-1 (Cav-1). This tumor suppressor directly regulates Ca^{2+} influx machineries and the Ca^{2+}-dependent apoptotic pathway. Cav-1 is a plasma membrane protein that also acts as a scaffolding protein for signaling molecules, including Ca^{2+}-signaling members and Ras. Ras proteins are GTPases activated by receptor tyrosine kinases that transduce extracellular signals to the nucleus. In 3T3NIH fibroblasts transformed with a retrovirus expressing the oncogenic H-Ras (H-Ras$_{12V}$), it was revealed that H-Ras$_{12V}$ accumulates at the MAM and plasma membrane-associated membranes (PAM), causing altered intracellular Ca^{2+} homeostasis. This compartmentalization

of H-Ras compromises the cooperation between MAM and PAM, promoting the loss of Cav-1 (Fig. 2) (56).

Another example includes BRAF mutations that can reprogram melanoma metabolism. Briefly, BRAF inhibitors (BRAFi) hamper glucose uptake prior to the complete elimination of melanoma tumor cells. BRAFi-treated melanomas rapidly became OXPHOS-dependent to survive. BRAFi induces increased mitochondrial activity and biogenesis, and an increased capacity for mitochondrial Ca^{2+} buffering associated with mitochondrial network remodeling. A close interaction between the ER and mitochondria in BRAFi-exposed melanoma facilitates mitochondrial Ca^{2+} uptake after its release from the ER that prevents ER-mediated cell death (93). Furthermore, the inhibition of Ca^{2+} transfer from the ER to mitochondria may preserve mitochondrial integrity and protect against Ca^{2+}-mediated apoptosis in human malignant pleural mesothelioma. A severe dysregulation of Ca^{2+} signaling is associated with resistance to apoptotic stimuli in mesothelioma cell lines and short-term cell cultures obtained from patients with malignant pleural mesothelioma (94).

The upregulation of some calcium transporters or their regulators has also been associated with resistance to apoptosis. Upregulation of MCU regulator 1 (MCUR1) has been associated with increased survival of liver cancer cells. MCUR1 overexpression indirectly inhibits mitochondrial-dependent intrinsic apoptosis due to AKT/MDM2 proto-oncogene-mediated p53 degradation by the elevated production of mitochondrial ROS (95). Another example includes TRPV6, which is a highly selective Ca^{2+} channel involved in prostate carcinogenesis. Increased expression of TRPV6 is strongly correlated with cancer progression and with the resistance to apoptosis observed in hormone-sensitive prostate cancer cells (Fig. 2) (96). These aforementioned studies refer to the failure of apoptotic machinery that may favor phenotypes that resist traditional chemotherapy treatments.

Autophagy is a conserved process for the delivery of cellular material to lysosomes for degradation. This process recycles molecules for the synthesis of proteins, nucleic acids, lipids and carbohydrates, or prevents accumulation of protein aggregates and damaged organelles, maintaining cellular homeostasis (30). Intracellular levels of calcium have also been identified as a potential regulator of autophagy. This process can be induced by rapamycin and Torin 1; both molecules selectively inhibit mTOR. Alternatively, starvation can induce autophagy; this process is more complex but it also involves mTOR and various upstream nutrient-sensing molecules, including AMPK and calcium/calmodulin-dependent protein kinase kinase β (CAMKK-β) (13). In HeLa cells, Ca^{2+} mobilizing agents (ionomycin, ATP and thapsigargin) stimulate autophagy via CAMKK, which directly activates AMPK to inhibit mTOR (97). In addition, Ca^{2+} signaling promotes increased mitochondrial fission that leads to autophagy in liver cancer cells (68).

Previous studies have reported that disruption of IP$_3$R-mediated Ca^{2+} delivery stimulates autophagy in various types of cancer (98,99). In this context, vesicle-associated membrane protein-associated protein B (VAPB), an integral ER protein, binds to the regulator of microtubule dynamics 3 (RMDN3), an OMM protein, to form one set of tethers. Under non-stressing

conditions, VAPB and RMDN3 overexpression favors the tether between the ER and mitochondria, thus inhibiting autophagy (Fig. 1); however, blocking the ER-mitochondria Ca^{2+} exchange using MCU-targeted antagonist siRNAs abrogates the effect of VAPB and RMDN3 overexpression, favoring autophagy. This highlights the role of VAPB-RMDN3 in ER-mitochondria Ca^{2+} delivery (13). Paradoxically, starvation-induced autophagy requires Ca^{2+} release from sensitized IP_3Rs (30), thus suggesting that the autophagy process depends on spatial and temporal parameters of Ca^{2+} signaling, in addition to nutrient status and the availability of growth factors (99). Autophagy favors cancer development due to the energetic supply provided by organelle degradation typically driven by this process, which allows tumor cells to become resistant to stressing conditions, such as anticancer therapies. Therefore, autophagy inhibition has emerged as a potential cancer treatment strategy (100).

10. Conclusions

Through several mechanisms, mitochondria serve an essential role in maintaining cellular calcium levels. These processes are meticulously regulated because calcium acts as a trigger of regulatory cascades that affect the mitochondria themselves and, in consequence, metabolism, migration and cell survival.

Tumor cells may remodel the systems that maintain cellular calcium homeostasis, in order to promote their survival and metastasis. Understanding the integral metabolic importance of Ca^{2+} signaling in tumor cells, which includes the participation of various organelles, will allow the development of numerous therapies that may benefit patients with cancer.

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SRG conceived, designed and wrote the manuscript. HPG was involved in writing and critically reviewing the manuscript, and figure design.

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Competing interests

The authors declare that they have no competing interests.

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