Endoplasmic Reticulum–Mitochondrial Ca\(^{2+}\) Fluxes Underlying Cancer Cell Survival

Hristina Ivanova, Martijn Kerkhofs, Rita M. La Rovere† and Geert Bultynck*†

Laboratory of Molecular and Cellular Signaling, Department of Cellular and Molecular Medicine, Leuven Kanker Instituut (LKI), KU Leuven, Leuven, Belgium

Calcium ions (Ca\(^{2+}\)) are crucial, ubiquitous, intracellular second messengers required for functional mitochondrial metabolism during uncontrolled proliferation of cancer cells. The mitochondria and the endoplasmic reticulum (ER) are connected via “mitochondria-associated ER membranes” (MAMs) where ER–mitochondria Ca\(^{2+}\) transfer occurs, impacting the mitochondrial biology related to several aspects of cellular survival, autophagy, metabolism, cell death sensitivity, and metastasis, all cancer hallmarks. Cancer cells appear addicted to these constitutive ER–mitochondrial Ca\(^{2+}\) fluxes for their survival, since they drive the tricarboxylic acid cycle and the production of mitochondrial substrates needed for nucleoside synthesis and proper cell cycle progression. In addition to this, the mitochondrial Ca\(^{2+}\) uniporter and mitochondrial Ca\(^{2+}\) have been linked to hypoxia-inducible factor 1\(\alpha\) signaling, enabling metastasis and invasion processes, but they can also contribute to cellular senescence induced by oncogenes and replication. Finally, proper ER–mitochondrial Ca\(^{2+}\) transfer seems to be a key event in the cell death response of cancer cells exposed to chemotherapeutics. In this review, we discuss the emerging role of ER–mitochondrial Ca\(^{2+}\) fluxes underlying these cancer-related features.

Keywords: cancer, endoplasmic reticulum–mitochondrial Ca\(^{2+}\) fluxes, Ca\(^{2+}\) signaling, inositol 1,4,5-trisphosphate receptor/Ca\(^{2+}\) channels, cell cycle regulation, mitochondrial metabolism, cell death signaling
cancer cells do not rely purely on glycolysis. The reprogrammed cellular metabolism in tumors also maintains sufficient levels of OXPHOS by using pyruvate generated by glycolysis. Indeed, the TCA cycle appears to complement glycolysis, supplying enough ATP, NADH, and biomass precursors for the biosynthesis of other macromolecules, like phospholipids and nucleotides (7). For instance, the TCA cycle intermediate oxaloacetate is used as a substrate for the biosynthesis of uridine monophosphate, a precursor of uridine-5’-triphosphate and cytidine triphosphate involving a rate-limiting step executed by dihydroorotase dehydrogenase, which, in turn, catalyzes the de novo synthesis of pyrimidines in the inner mitochondrial membrane (8). Its dehydrogenase activity depends on the electron transport chain (ETC), where it feeds the electrons of the dihydroorotate oxidation to the ETC by reducing respiratory ubiquinone. Thus, adequate ETC activity and proper pyrimidine biosynthesis are intimately linked (8).

**MITOCHONDRIAL Ca²⁺ SIGNALS AS REGULATORS OF CELL DEATH AND SURVIVAL**

Ca²⁺, a cofactor of several rate-limiting TCA enzymes [pyruvate-, isocitrate-, and α-ketoglutarate dehydrogenases (PDH, IDH, and αKGDH)], plays a pivotal role in the regulation of mitochondrial metabolism and bioenergetics (9). As such, Ca²⁺ present in the mitochondrial matrix is required for sufficient NADH and ATP production (10).

**Transfer of Ca²⁺ Signals from the Endoplasmic Reticulum (ER) to the Mitochondria**

The accumulation of Ca²⁺ into the mitochondria strictly depends on the ER, which serves as the main intracellular Ca²⁺-storage organelle. Ca²⁺ is stored in the ER by the action of ATP-driven sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) with SERCA2b (11) as the housekeeping isoform and by ER luminal Ca²⁺-binding proteins like calreticulin and calnexin (12). Ca²⁺ can be released from the ER via intracellular Ca²⁺-release channels, including inositol 1,4,5-trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs). IP₃Rs, which are activated by the second messenger IP₃, are ubiquitously expressed in virtually all human cell types (13, 14). IP₃ is produced through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC)β/γ, an enzyme activated in response to hormones, neurotransmitters, and antibodies. IP₃ activity can be suppressed by compounds like xestospongin B (15), which directly inhibits IP₃Rs, or U73122, which inhibits PLC activity (16). Although 2-APB (17) and xestospongin C (18) are also used as IP₃R inhibitors, these compounds affect other Ca²⁺-transport systems. For instance, 2-APB is known to inhibit store-operated Ca²⁺ entry through Orai1 (19) and SERCA (20), and to activate Orai3 channels (19). In addition, similarly to its analogs like DPB162-AE, 2-APB can induce a Ca²⁺ leak from the ER, partially mediated by ER-localized Orai3 channels (20–23). Xestospongin C also inhibits SERCA with a potency that is equal to its inhibitory action on IP₃Rs (24). RyRs are predominantly expressed in excitable cells, including several muscle types, neuronal cells, and pancreatic β cells (25). In most cells, RyRs are mainly activated by cytosolic Ca²⁺ via Ca²⁺-induced Ca²⁺ release, while in skeletal muscle they are activated through a direct coupling with the dihydropyridine receptor upon depolarization (26). RyR activity can be counteracted by dantrolene (27) and high concentrations of ryanodine (28).

The efficient Ca²⁺ exchange between the ER and the mitochondria takes place in specialized microdomains, which are established by organellar contact sites and which can be isolated biochemically as mitochondria associated-ER membranes (MAMs) (29–31). Several proteins are involved in ER–mitochondrial tethering, including IP₃Rs at the ER side and the Ca²⁺-permeable channels voltage-dependent anion channel type 1 (VDAC1) at the mitochondrial side (32, 33). The Ca²⁺ released through IP₃Rs and eventually transferred to the mitochondrial intermembrane space by VDAC1 accumulates in the mitochondrial matrix via the mitochondrial Ca²⁺ uniporter (MCU). The functional properties of the MCU are tightly regulated by a growing list of interacting proteins, which enable a tight control over the Ca²⁺ levels in the mitochondrial matrix (34). These MCU modulators have an important cell physiological impact on mitochondrial metabolism, cell survival, and cell death (35–44).

Seminal work using aequorin targeted to the mitochondria revealed that IP₃-evoked Ca²⁺ signals were efficiently transferred into the mitochondria even when IP₃-induced cytosolic Ca²⁺ concentration ([Ca²⁺]cyt) rises were relatively small (45). In contrast, artificial rises in [Ca²⁺]er, were ineffective in increasing mitochondrial [Ca²⁺] ([Ca²⁺]mit). This indicated that local [Ca²⁺]er can be very high in the proximity of IP₃R channels, which is then sensed by the mitochondria, leading to mitochondrial Ca²⁺ accumulation. These findings were underpinned by perimitochondrial [Ca²⁺] measurements, showing that the local [Ca²⁺]mit was about 20-fold higher than global [Ca²⁺]er, allowing a “quasi-synaptic” transmission of the Ca²⁺ signal from the ER into the mitochondrial matrix (46). More precise determinations of local [Ca²⁺]er at the ER–mitochondrial contact sites were obtained with pericam-tagged ligands, which indicated concentrations of ~10 μM (47). Importantly, mitochondrial Ca²⁺ transfer from the ER critically depended on IP₃R-mediated Ca²⁺ release, since thapsigargin-induced depletion of the ER, which occurs via ER Ca²⁺-leak channels that are spread out over the ER membrane, was ineffective in eliciting a [Ca²⁺]mit rise (46). Efficient IP₃R-mediated Ca²⁺ transfer into the mitochondria is achieved by the molecular chaperone 75-kDa glucose-regulated protein (GRP75), which physically links IP₃Rs to VDAC1 within the MAMs (32). Knockdown of GRP75 impairs the IP₃R-mediated Ca²⁺ transfer to the mitochondria (32).

A positive feedback between the Ca²⁺ transfer from the ER to the mitochondria and the formation of H₂O₂ nanodomains at the ER–mitochondrial interface has recently been described (48). These H₂O₂ nanodomains are formed upon physiological stimulation of the IP₃R-mediated Ca²⁺ transfer to the mitochondria. Ca²⁺ fuels the ETC, whose functionality determines the production of H₂O₂. In addition, Ca²⁺ accumulation in the matrix induced K⁺ flux, which results in drastically reduced volume of the cristae.
and subsequent H$_2$O$_2$ flux into the mitochondrial matrix, thereby increasing the mitochondrial matrix volume and squeezing out H$_2$O$_2$-rich fluid at the ER–mitochondrial interface. This locally released H$_2$O$_2$ sensitizes IP$_3$Rs to low concentrations of agonists and stimulates Ca$^{2+}$ oscillations, thereby further boosting mitochondrial bioenergetics.

### Translation of the Ca$^{2+}$ Signals in the Mitochondria: Cell Survival, Autophagy, or Apoptosis

The adequate transfer of Ca$^{2+}$ from the ER into the mitochondria requires a proper filling of the ER Ca$^{2+}$ stores. Also, luminal Ca$^{2+}$ controls IP$_3$-mediated Ca$^{2+}$ release (49). Thus, lowering of the ER Ca$^{2+}$ levels ([Ca$^{2+}]_{\text{ER}}$) will dampen ER–mitochondrial Ca$^{2+}$ transfer, while increasing [Ca$^{2+}]_{\text{ER}}$ will augment ER–mitochondrial Ca$^{2+}$ transfer. As a consequence, changes in the ER Ca$^{2+}$-store content will eventually impact the level of Ca$^{2+}$ transfer from the ER to the mitochondria and thus eventually cell death and survival decisions. A lowering of the ER Ca$^{2+}$-store content has been shown to serve as a survival mechanism exploited by several pro-survival proteins and oncogenes, including Bax inhibitor-1 (BI-1), antiapoptotic Bcl-2 proteins, and Ras (50–53) as extensively described in Ref. (54). This does not only render cells more resistant to apoptotic triggers but it may also facilitate the survival of damaged or stressed cells, thereby resulting in oncogenesis and cancer cell survival. Several mechanisms can account for this [Ca$^{2+}]_{\text{ER}}$ lowering, including the function of BI-1 as an ER Ca$^{2+}$-leak channel (55) and the sensitization of other ER Ca$^{2+}$ channels that contribute to the ER Ca$^{2+}$ leak, like IP$_3$Rs (52, 56).

The decrease in [Ca$^{2+}]_{\text{ER}}$ can either induce autophagy due to an impaired basal mitochondrial Ca$^{2+}$ transfer (10) or suppress autophagy due to diminished [Ca$^{2+}]_{\text{mito}}$ increases (57). On the one hand, low [Ca$^{2+}]_{\text{ER}}$ results in decreased spontaneous activity of IP$_3$Rs, thereby abrogating its positive effect on the mitochondrial metabolism and resulting in the activation of AMP-activated kinase (AMPK) and subsequent increase in autophagic flux (10). Indeed, in many cells, IP$_3$Rs appear to be constitutively active, thereby feeding Ca$^{2+}$ in the mitochondria, which is necessary for mitochondrial metabolism (Figure 1). This is supported by

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** | Endoplasmic reticulum (ER)–mitochondrial Ca$^{2+}$ transfers in cancer hallmarks. ER-mitochondrial Ca$^{2+}$ transfers will impact several hallmarks of cancer. First, ER-originating, inositol 1,4,5-trisphosphate receptor (IP$_3$R)-driven Ca$^{2+}$ signals delivered to the mitochondria will drive the tricarboxylic acid (TCA) cycle, which will not only result in ATP production via NADH and the electron transport chain but also in the production of mitochondrial substrates shuttled to biosynthetic pathways for macromolecules like nucleosides. This is accompanied by a decrease in autophagic flux due to a low activity of AMP-activated kinase. Second, mitochondrial Ca$^{2+}$ signals will also increase mitochondrial reactive oxygen species (ROS) production, which will drive the transcription of the mitochondrial Ca$^{2+}$ uniporter (MCU) regulates breast cancer progression via hypoxia-inducible factor 1α (HIF1α)—target genes with functions in metabolic reprogramming and, metastasis and invasion. Third, ER–mitochondrial Ca$^{2+}$ fluxes are involved in mediating cellular senescence induced by oncogenes and replication. The mechanism involves the partial depolarization of the mitochondrial potential ($\Delta \psi_m$) and accumulation of ROS. Fourth, ER–mitochondrial Ca$^{2+}$ fluxes impact cellular sensitivity toward apoptotic stimuli. In particular, mitochondrial Ca$^{2+}$ overload, together with the accompanying ROS production, has been a critical factor for mitochondrial permeability transition pore (mPTP) opening. Thus, the cell death-inducing properties of several chemotherapeutics actually critically depend on their ability to elicit mitochondrial Ca$^{2+}$ overload. Thus, ER–mitochondrial Ca$^{2+}$ transfers display both oncogenic properties (cell cycling, proliferation, metabolic reprogramming, metastasis, and invasion) and tumor suppressive properties (reduced autophagy and increased cell death sensitivity).
observations made in DT40 B-lymphocytes in which all three IP₃Rs isofoms have been deleted. These cells display a decreased mitochondrial NADH and ATP production due to a decreased activity of Ca²⁺-dependent dehydrogenases, the F1F0-ATPase, and the ETC (9, 58, 59). The decline in ATP levels results in the activation of AMPK, which inhibits the mammalian target of rapamycin (mTOR). In addition to mTOR suppression, AMPK promotes autophagy also through phosphorylation of unc-51-like kinase 1 (ULK1) and activation of the ULK1 complex (60, 61). However, the AMPK-dependent induction of autophagy upon inhibition of ER–mitochondrial Ca²⁺ transfer was shown to be mTOR-independent, suggesting a prominent role for the AMPK–ULK1 axis in this paradigm (10). Of note, while [Ca²⁺]ₘ rises appear to suppress autophagy (62), [Ca²⁺]ᵥ rises have been implicated in autophagy induction by the activation of calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), an upstream activator of AMPK. Thus, low [Ca²⁺]ᵢₐ can sustain autophagy by diminishing [Ca²⁺]ᵥ. It was proposed that antia apoptotic Bcl-2, by lowering the ER Ca²⁺ levels, could suppress cytosolic Ca²⁺ signals evoked by various pharmacological and physiological agents, thereby counteracting the activation of CaMKKβ-controlled autophagy (57, 63).

Further complexity of autophagy regulation arises from the fact that IP₃R sensitization by accessory proteins might have an opposite outcome on autophagy, dependent on whether the sensitization is limited to MAMs or whether it occurs all over the ER membrane. Indeed, IP₃R sensitization in the MAMs would lead to increased basal mitochondrial Ca²⁺ delivery, driving ATP production and thus suppressing autophagy. For example, Bcl-XL, which is present in the MAMs, can augment mitochondrial metabolism and is able to reduce autophagy by local IP₃R sensitization in the MAMs (64, 65) (Table 1). In contrast, IP₃R sensitization outside the MAMs will affect the overall ER Ca²⁺ loading due to an increased ER Ca²⁺ leak through IP₃Rs that become sensitive to basal IP₃ levels. This would result in partially depleted ER Ca²⁺ stores and decreased basal mitochondrial Ca²⁺ delivery, leading to reduced ATP production and increased autophagy. For example, BI-1, which presumably is ubiquitously present in the ER membrane, reduces the steady-state ER Ca²⁺ levels through IP₃R sensitization, decreasing mitochondrial bioenergetics and thus inducing autophagy (66).

In contrast to the reduced mitochondrial Ca²⁺ supply, which triggers autophagy, it has become clear that excessive Ca²⁺ transfer from the ER to the mitochondria results in cell death (83–85) (Figure 1). This involves the opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane, resulting in mitochondrial swelling and mitochondrial membrane rupture, eventually leading to cytochrome c release and apoptosis, if sufficient levels of ATP are available (85). Many cell death-inducing agents, like H₂O₂ (86, 87), arachidonic acid (88), ceramide (50, 86), and menadione (89, 90) have been shown to act at the ER by triggering Ca²⁺ release through IP₃Rs and subsequently provoking mitochondrial Ca²⁺ rises (91). Moreover, the ability of chemotherapeutics, like adriamycin (77), arsenic trioxide (71), and mitotane (82) and of photodynamic therapy (78) to kill cancer cells strongly depends on their ability to adequately induce ER–mitochondrial Ca²⁺ transfer (92). The spectrum of chemotherapeutics acting in this way might be quite broad, since recently it was shown that cisplatin and topotecan increase [Ca²⁺]ᵥ over time, although [Ca²⁺]ₘ was not determined (93). The transfer of pro-apoptotic Ca²⁺ signals to the mitochondria appears to be mediated by VDAC1 and not VDAC2 or VDAC3 (86). Also further insights in the mechanism underlying mPTP opening upon mitochondrial Ca²⁺ overload have been obtained. Ca²⁺ accumulating in the mitochondrial matrix binds to cardiolipin, which dissociates from the respiratory chain complex II and eventually results in its disassembly. The unleashed subunits of complex II produce reactive oxygen species (ROS) in the mitochondrial matrix, resulting in the opening of the mPTP (94).

The dichotomous impact of mitochondrial Ca²⁺ on both apoptosis and autophagy implies that reduced mitochondrial Ca²⁺ transfer will simultaneously result in acquired resistance to apoptotic stimuli and in increased autophagy (Figure 1) (95). This mechanism has been shown to be responsible for the sustained proliferation of cells deficient in promyelocytic leukemia protein (PML), a tumor suppressor present at the MAMs that augments ER–mitochondrial Ca²⁺ flux on the one hand and excessive chemotherapeutic resistance on the other hand (71, 72). Indeed, loss of PML reduced basal ER–mitochondrial Ca²⁺ transfers, thereby inducing sustained autophagy, promoting malignant cell survival and reduced chemotherapy-induced apoptosis contributing to poor chemotherapeutic efficacy (Table 1).

Finally, it is important to remark that cell death and survival are regulated by mitochondrial dynamics, including mitochondrial fusion, mainly mediated by optic atrophy 1 and by dynamin-related GTPases mitofusin-1 (Mfn-1) and Mfn-2, and mitochondrial fission, mainly mediated by the cytosolic soluble dynamin-related protein 1 (Drp1) (96, 97). Mitochondrial fragmentation leads to Bax-dependent apoptosis, while hyperfusion of mitochondria in response to a decline in Drp1 results in proliferation. Moreover, mitochondrial dynamics themselves are also regulated by Ca²⁺ signaling via calcineurin-mediated dephosphorylation of Drp1 (98). Mitochondrial hyperfusion may also render cells more sensitive to apoptotic stimuli due to hyperpolarization of the mitochondrial membrane and thus an increased driving force for mitochondrial Ca²⁺ uptake (99, 100). Hyperpolarization of mitochondrial membrane is also tightly connected to ROS production and release. As such, extensive ROS generation results in hyperpolarization of the mitochondrial membrane, followed by amplified ROS generation. ROS are released into the cytosol, where they can affect other mitochondria. This process is called ROS-induced ROS release and it could play important role in mitochondrial and cellular injuries (101).

## CANCER CELLS’ ADDICTION TO CONSTITUTIVE ER–MITOCHONDRIAL Ca²⁺ SIGNALING

Clearly, basal IP₃,R-driven Ca²⁺ signals and subsequent ER–mitochondrial Ca²⁺ transfer impact cell death and survival processes. Inhibition of IP₃Rs and thus spontaneous Ca²⁺ signals lead to reduced mitochondrial bioenergetics and increased autophagy, allowing cell survival (10). Recently, the role of basal
| Protein | Modulator | Mechanism | ER–mitochondrial \(\text{Ca}^{2+}\) flux | Cellular/in vivo effect | Model | Reference |
|---------|-----------|-----------|-----------------------------------|-----------------------|-------------------|------------|
| IP_{3}R | IP_{3}R1/IP_{3}R3 knockdown | IP_{3}R1/IP_{3}R3 expression ↓ | ↓ Cell death ↑ caused by mitotic catastrophe | HrasG12V-cyclin-dependent kinase 4 (CDK4)-transformed human fibroblasts, tumorigenic cell lines: breast, prostate, and cervix (HeLa) | Primary fibroblasts; non-tumorigenic breast and prostate cell lines | (67) |
|         | XeB      | Selective IP_{3}R inhibitor | | | | |
|         | U73122   | PLC inhibitor (IP_{3}) | | | | |
| IP_{3}R  | IP_{3}R2/IP_{3}R3 knockdown | IP_{3}R2/IP_{3}R3 expression ↓ | ↓ Cell death ↑ caused by excessive autophagy | Breast cancer cell line (MCF-7) | B16F10 melanoma cell xenograft (only performed with XeB) | (68) |
|         | XeC/2-APB | Non-selective IP_{3}R inhibitors | | | | |
| IP_{3}R  | IP_{3}R2 knockdown | IP_{3}R2 expression ↓ | ↓ Escape from oncogene-induced senescence | Immortalized human mammary epithelial cells (HECs) | | (69) |
|         | BI-1     | (Direct) sensitization of IP_{3}R | ↓ Autophagy ↑ | HeLa and MEF cells | | (56, 66) |
|         | Bcl-XL   | Direct sensitization of IP_{3}Rs (at the MAMs), promoting pro-survival \(\text{Ca}^{2+}\) oscillations | ↑ Cellular bioenergetics ↑ | Reconstituted DT40-triple IP_{3}R knockout cells | CHO cells | (64, 65, 70) |
|         | PML      | Counteracting Akt-mediated IP_{3}R3 phosphorylation through PP2A recruitment | ↑ Apoptosis ↓ | MEF cells | H1299 | (71, 72) |
|         | Bcl-2    | Direct inhibition of IP_{3}Rs | ↓ Apoptosis ↓ | APL NB4 cells | WEHI7.2 cells and Jurkat | (54, 73, 74) |
|         | H_{2}O_{2} | Direct sensitization of IP_{3}Rs (at the MAMs), via oxidation of specific thiol group of IP_{3}R | ↑ Cellular bioenergetics ↑ | HEPG2 | | (48) |
|         |          | Functioning as an ER \(\text{Ca}^{2+}\)-leak channel, mainly outside the mitochondria-associated ER membranes (MAMs) | | | | |
| SERCA   | TMX1     | Binds and inhibits SERCA2b (at the MAMs) in a calnexin-dependent manner | ↑ Apoptosis ↓ | A375P melanoma and HeLa cell (xenograft) | | (75, 76) |
|         | p53 (extranuclear) | Accumulates at the ER and MAMs upon chemotherapy treatment, directly binding and activating SERCA2b by changing its oxidative state | ↑ Apoptosis ↑ | MEF, HeLa, and H1299 (human non-small cell lung carcinoma cell line) | HCT-116 and MDA-MB 468 | (77, 78) |
|         | Resveratrol | Reduced SERCA activity due to inhibition of mitochondrial ATP synthase | ↑ Apoptosis ↑ | Endothelial/epithelial cancer cell hybrid EA.hy926, HeLa | | (79) |
| VDAC    | Mcl-1    | Binds and activates voltage-dependent anion channel type 1 (VDAC1) and VDAC3 | ↑ Cell migration ↑ caused by mitochondrial ROS ↑ | NSCLC cell lines | | (80) |
| MCU     | MCU knockdown | MCU expression ↓ | ↓ Metastatic cell motility and matrix invasiveness ↓ caused by decreased mitochondrial ROS and HIF1-mediated transcription | Triple-negative breast cancers | MDA-MB-231 xenografts | (81) |
|         |          | Cell death ↑ caused by mitotic catastrophe | | | | |
| FATE1   |          | Uncoupling of ER and mitochondria | ↓ Apoptosis ↓ | Adrenocortical carcinoma cells | | (82) |

*aSarco/endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA) inhibitor.

bStore-operated \(\text{Ca}^{2+}\) entry and SERCA inhibitor.

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**TABLE 1** | The impact of experimental, physiological, and cancer-related modulators of endoplasmic reticulum (ER)–mitochondrial \(\text{Ca}^{2+}\) flux on cell death, survival, and migration.

**Protein** | **Modulator** | **Mechanism** | **ER–mitochondrial \(\text{Ca}^{2+}\) flux** | **Cellular/in vivo effect** | **Model** | **Reference** |
|------------|--------------|---------------|-----------------------------------|-----------------------|-------------------|------------|
| IP_{3}R    | IP_{3}R1/IP_{3}R3 knockdown | IP_{3}R1/IP_{3}R3 expression ↓ | ↓ Cell death ↑ caused by mitotic catastrophe | HrasG12V-cyclin-dependent kinase 4 (CDK4)-transformed human fibroblasts, tumorigenic cell lines: breast, prostate, and cervix (HeLa) | Primary fibroblasts; non-tumorigenic breast and prostate cell lines | (67) |
| XeB       | Selective IP_{3}R inhibitor | | | | | |
| U73122    | PLC inhibitor (IP_{3}) | | | | | |
| IP_{3}R    | IP_{3}R2/IP_{3}R3 knockdown | IP_{3}R2/IP_{3}R3 expression ↓ | ↓ Cell death ↑ caused by excessive autophagy | Breast cancer cell line (MCF-7) | B16F10 melanoma cell xenograft (only performed with XeB) | (68) |
| XeC/2-APB | Non-selective IP_{3}R inhibitors | | | | | |
| IP_{3}R    | IP_{3}R2 knockdown | IP_{3}R2 expression ↓ | ↓ Escape from oncogene-induced senescence | Immortalized human mammary epithelial cells (HECs) | | (69) |
| BI-1      | (Direct) sensitization of IP_{3}R | ↓ Autophagy ↑ | HeLa and MEF cells | | (56, 66) |
| Bcl-XL    | Direct sensitization of IP_{3}Rs (at the MAMs), promoting pro-survival \(\text{Ca}^{2+}\) oscillations | ↑ Cellular bioenergetics ↑ | Reconstituted DT40-triple IP_{3}R knockout cells | CHO cells | (64, 65, 70) |
| PML       | Counteracting Akt-mediated IP_{3}R3 phosphorylation through PP2A recruitment | ↑ Apoptosis ↓ | MEF cells | H1299 | (71, 72) |
| Bcl-2     | Direct inhibition of IP_{3}Rs | ↓ Apoptosis ↓ | APL NB4 cells | WEHI7.2 cells and Jurkat | (54, 73, 74) |
| H_{2}O_{2} | Direct sensitization of IP_{3}Rs (at the MAMs), via oxidation of specific thiol group of IP_{3}R | ↑ Cellular bioenergetics ↑ | HEPG2 | | (48) |
| SERCA     | TMX1       | Binds and inhibits SERCA2b (at the MAMs) in a calnexin-dependent manner | ↑ Apoptosis ↓ | A375P melanoma and HeLa cell (xenograft) | | (75, 76) |
| p53 (extranuclear) | Accumulates at the ER and MAMs upon chemotherapy treatment, directly binding and activating SERCA2b by changing its oxidative state | ↑ Apoptosis ↑ | MEF, HeLa, and H1299 (human non-small cell lung carcinoma cell line) | HCT-116 and MDA-MB 468 | (77, 78) |
| Resveratrol | Reduced SERCA activity due to inhibition of mitochondrial ATP synthase | ↑ Apoptosis ↑ | Endothelial/epithelial cancer cell hybrid EA.hy926, HeLa | | (79) |
| VDAC      | Mcl-1      | Binds and activates voltage-dependent anion channel type 1 (VDAC1) and VDAC3 | ↑ Cell migration ↑ caused by mitochondrial ROS ↑ | NSCLC cell lines | | (80) |
| MCU       | MCU knockdown | MCU expression ↓ | ↓ Metastatic cell motility and matrix invasiveness ↓ caused by decreased mitochondrial ROS and HIF1-mediated transcription | Triple-negative breast cancers | MDA-MB-231 xenografts | (81) |
|         |          | Cell death ↑ caused by mitotic catastrophe | | | | |
| FATE1     |          | Uncoupling of ER and mitochondria | ↓ Apoptosis ↓ | Adrenocortical carcinoma cells | | (82) |
IP₃R-mediated Ca²⁺ signaling and ER–mitochondrial Ca²⁺ transfer for cancer cell survival was investigated in more detail (67). A comparison was made between non-tumorigenic and tumorigenic cell lines, as well as between non-transformed primary human fibroblasts and fibroblasts transformed by the ectopic expression of oncogenic HRasG12V and cyclin-dependent kinase 4. For reasons of clarity, we will refer to the former as “normal cells” and to the latter as “cancer cells.” Strikingly, inhibition of IP₃R activity, knockdown of IP₃R or MCU led in both normal and cancer cells to a so-called “bioenergetic crisis” characterized by a decreased basal and maximal oxygen consumption rate and increased AMPK phosphorylation, subsequently resulting in an increased autophagic flux. However, these interventions resulted in cell death in the cancer cells but not in the normal cells, indicating that autophagy upregulation induced upon IP₃R inhibition was sufficient to sustain cell survival in the normal cells but not in cancer cells (67). Similar results were obtained in another recent study, which also implicated autophagy induced by IP₃R inhibition in cancer cell death (68). Selective knockdown of IP₃R isoforms using siRNA or general IP₃R inhibition using 2-APB or xestospongin C compromised mitochondrial bioenergetics, led to generation of ROS, activation of AMPK, and upregulation of Atg5, an essential autophagy gene. This resulted in excessive autophagy in the cancer cells. Cells could be rescued by ROS scavengers and autophagy inhibitors, indicating that autophagy was at least in part responsible for the cell death. 2-APB was also used in xenograft models, where it strongly suppressed in vivo tumor growth. It is important to note that 2-APB and xestospongin C cannot be considered as selective inhibitors of IP₃Rs and thus their impact on cancer cell survival might be related to off-target effects (Table 1).

However, the fact that in some conditions autophagy upregulation is not sufficient for cancer cell survival upon IP₃R inhibition is in striking contrast to the important role of autophagy for cancer cell survival in conditions of nutrient starvation (102). Ras-driven lung cancer cells were dependent on autophagy for their survival during starvation conditions. Consistent with this, caloric restriction was more effective to suppress Ras-driven tumor growth when it was combined with autophagy inhibition (103). This may indicate that the contribution of autophagy for cancer cell survival might be different dependent on the way autophagy was induced (IP₃R inhibition versus starvation), which may be due to differences in the produced breakdown products and their usage in metabolic and biosynthetic pathways (104). The cancer cell death induced by IP₃R inhibition could be rescued by providing the cells with cell-permeable mitochondrial substrates like methyl pyruvate, that is oxidized to NADH necessary to drive OXPHOS and production of ATP or dimethyl α-ketoglutarate, a precursor for glutamine to fuel the TCA cycle, where it enters and is oxidized by a Ca²⁺-dependent α-KGDH as the first step (67). Moreover, the protective effects of the substrates in xestospongin B-treated cancer cells were unrelated to their antioxidant properties, since the antioxidant N-acetyl-cysteine could not protect the cancer cells against cell death. Instead, nucleoside complementation could rescue the death of the cancer cells induced by IP₃R inhibition (67), indicating that constitutive ER–mitochondrial Ca²⁺ fluxes are required for cancer cell survival by sustaining an adequate source of mitochondrial substrates for nucleotide synthesis. This phenomenon was also observed in vivo, where tumor growth could be reduced upon treatment with xestospongin B.

Indeed, in conditions of suppressed ER–mitochondrial Ca²⁺ flux, normal cells display slower cell-cycle progression and become arrested at the G1/S checkpoint. This prevents DNA synthesis and shifts cells to accumulate in the G1 phase rather than the S phase (Figure 2), ultimately reducing the rate of daughter cell generation and proliferation. Conversely, cancer cells exposed to IP₃R inhibitors have lost proper control over their G1/S checkpoint, progressing through the cell cycle and undergoing mitosis irrespective of their OXPHOS and mitochondrial bioenergetic status. As such, cancer cells will divide even though their mitochondrial metabolism is insufficient to cope with the anabolic pathways needed to make a living daughter cell, eventually resulting in a “mitotic catastrophe” upon daughter cell separation (67). Interestingly, arresting cancer cells in the G1/S phase and preventing them to undergo mitosis strongly suppressed cell death induced by IP₃R inhibition. Hence, beyond the well-established roles of IP₃Rs in apoptosis, these data reveal that, in the absence of proper cell-cycle control, cells are addicted to constitutive IP₃R function and sustained ER–mitochondrial Ca²⁺ transfer for fueling mitochondrial metabolism. These ER–mitochondrial Ca²⁺ fluxes maintain sufficiently high levels of TCA cycling by ensuring the activity of Ca²⁺-dependent dehydrogenases, thereby delivering an adequate supply of mitochondrial substrates required for nucleotide production and DNA synthesis during ongoing proliferation (67).

Interestingly, the need for adequate mitochondrial Ca²⁺ signaling in tumor cells is further supported by a very recent study performed in triple-negative breast cancer (81). It was shown that MCU expression positively correlated with the metastatic phenotype and clinical stage of the breast cancers, while the expression of MCUb, a negative regulator of MCU (37), displayed a negative correlation. Strikingly, silencing of MCU blunted cell invasiveness without affecting cell viability. The in vivo growth of breast cancer cells in which MCU was deleted was severely impaired, correlating with an altered cellular redox state and impaired mitochondrial production of ATP. In this mechanism, MCU-mediated Ca²⁺ uptake in the mitochondria resulted in increased ROS production and activation of hypoxia-inducible factor 1α signaling, contributing to tumor growth and metastatic behavior (81). Similar results were reported in NSCLS cells where mitochondrial ROS generation and increased cell migration were correlating to enhanced [Ca²⁺]mit uptake through Mcl-1/VDAC interaction (80) (Table 1).

Further studies are necessary in order to determine how cancer cells escape from the G1/S checkpoint with impaired mitochondrial bioenergetics due to reduced ER–mitochondrial Ca²⁺ fluxes. However, an important link between mitochondrial dynamics and cell-cycle control was described (106). This study revealed that at the G1/S checkpoint the mitochondrial structure changes into a single tubular network, electrically coupled and hyperpolarized, boosting ATP production (Figure 3) (106). The progression of the cell cycle is ensured by specific cyclins associated with CDKs (107). The G1-to-S transition, which ensures the initiation of DNA replication, is controlled by cyclin E, which,
Cancer cells are addicted to endoplasmic reticulum (ER)-mitochondrial Ca\textsuperscript{2+} fluxes to produce tricarboxylic acid (TCA)-dependent mitochondrial substrates used to sustain their uncontrolled proliferation. In both non-malignant and malignant cells, mitochondria require Ca\textsuperscript{2+} from the ER Ca\textsuperscript{2+} store for an adequate performance of the TCA cycle, which ultimately leads to energy production (ATP), redox homeostasis (NADH), and anabolism, e.g., of pyrimidine and purine nucleotides. The Ca\textsuperscript{2+}-dependent control of the TCA cycle is due to the Ca\textsuperscript{2+}-dependent activity of several rate-limiting enzymes (PDH, ICDH, and αKGDH, all indicated in red). Ca\textsuperscript{2+} is efficiently delivered to the mitochondria in a quasi-synaptic manner involving Ca\textsuperscript{2+}-signaling microdomains established at mitochondria-associated ER membranes involving inositol 1,4,5-trisphosphate receptor (IP\textsubscript{3}R), voltage-dependent anion channel type 1 (VDAC1), and mitochondrial Ca\textsuperscript{2+} uniporter (MCU) as Ca\textsuperscript{2+}-transport systems. Of note, although cancer cells switch to glycolysis for their ATP production, they too rely on functional mitochondria for the production of mitochondrial substrates used for anabolic processes, like the generation of nucleotides required for the DNA synthesis necessary for their deregulated cell cycle progression and proliferation. Ablation of these ER-mitochondrial Ca\textsuperscript{2+} fluxes (e.g., by using siRNA-based approaches or pharmacological inhibitors like xestospongin B) results in compromised mitochondrial bioenergetics, causing a decline in ATP, NADH, and nucleotides. In both non-malignant and malignant cells, this leads to an increase in AMPK activity. However, in non-malignant cells, increased AMPK activity will result in an arrest at the G1/S checkpoint, likely involving p53 activation and cyclin E downregulation, which will dampen proliferation as a cell survival strategy. In malignant cells, the link between AMPK activity and the G1/S checkpoint is lost (e.g., due to p53 mutations or cyclin deregulation). As a consequence, despite the mitochondrial bioenergetic crisis and the lack of mitochondrial substrates for DNA synthesis, cancer cells will progress toward the S phase and mitosis. This results in necrotic cell death due to mitotic catastrophe. This figure was originally published in Ref. (105). © 2016 Geert Bultynck. A copyright license to republish this figure has been obtained.

In turn, further binds and activates CDK2 to phosphorylate downstream targets for DNA production. Cyclin E abundance is restricted to the transition from the G1 phase to the S phase and decreases with the progression of the cell cycle. Mitochondrial hyperfusion will support ATP production and as such cyclin E stability, enabling S-phase progression. This actually establishes an important "mitochondrial checkpoint" that only permits G1/S progression when mitochondrial bioenergetics and cellular health are adequate (Figure 2). Based on the model proposed by Finkel and Hwang (108), cells with impaired mitochondrial bioenergetics and, thus reduced ATP output and increased AMPK activity, will activate p53 and p21, a cell cycle regulator, leading to a drop in cyclin E (109) and an arrest of the cells at the G1 phase due to their inability to overcome the G1/S checkpoint (Figure 3). In light of the requirement for a burst of ATP production for proper S phase progression, an increased mitochondrial Ca\textsuperscript{2+} demand would also be expected. Therefore, further work is required to establish whether IP\textsubscript{3}R activity and ER-mitochondrial tethering and/or Ca\textsuperscript{2+} transfers could become enhanced at the G1/S transition to support this increased ATP production as part of the "mitochondrial checkpoint." Previous studies have implicated IP\textsubscript{3}R sensitization as a critical step during G1/S transition and identified IP\textsubscript{3}Rs as targets for cyclins and substrates for CDKs (110). However, in cancer cells, the G1/S checkpoint control appears to be lost despite the fact that IP\textsubscript{3}R inhibition still leads to activation of AMPK, implying defects in the mechanisms linking AMPK to the G1/S checkpoint arrest, for example, mutations impairing p53 activity or hyperactivating CDKs. Previous work indicated that p53 mutations could result in a bypass of G1/S arrest (108). Thus, re-expression of p53 may restore the G1/S checkpoint control in a number of these cancer cell types exposed to IP\textsubscript{3}R inhibition, thereby slowing down cell cycle progression and proliferation and preventing cell death by mitotic catastrophe.
A boost in mitochondrial ATP output provides a mitochondrial checkpoint for cellular health, enabling cells to bypass the G1/S checkpoint and cell cycle progression. Based on Mitra et al. (106), mitochondrial structure changes along the cell cycle progression. At the late G1 phase, the mitochondrial structure changes into a giant, single tubular network, electrically coupled and hyperpolarized, boosting ATP production. The G1–S transition that ensures the initiation of DNA replication is controlled by the cyclin E, which, in turn, further binds and activates CDK2. Cyclin E is upregulated upon increased ATP output, enabling S-phase progression and proliferation. Non-tumorigenic cells experiencing a reduction of ATP production due to compromised mitochondrial bioenergetics will trigger the G1/S checkpoint arrest due to AMP-activated kinase (AMPK) activation and subsequent phosphorylation and activation of the tumor suppressor protein p53 that in turn downregulates cyclin E protein levels. In tumorigenic cells, it is anticipated that this tight link between adequate mitochondrial bioenergetics and the G1/S checkpoint is lost. Hence, cancer cells can progress through the cell cycle irrespective of their mitochondrial bioenergetic status. Thus, a mitochondrial bioenergetic crisis will slow down the cell cycle and proliferation in normal cells, while in cancer cells, the cell cycle will continue, eventually resulting in a mitotic catastrophe.

In addition to the addiction of some cancer cells to constitutive ER–mitochondrial Ca2+ fluxes, ER–mitochondrial contact sites and Ca2+-signaling events might be altered to favor cancer cell survival. This concept is supported by another recent study. TMX1, a redox-sensitive oxidoreductase that is enriched in the MAMs in a palmitoylation-dependent manner, was shown to regulate mitochondrial bioenergetics and in vivo tumor growth by controlling ER–mitochondrial Ca2+ signaling (75, 76). Upon palmitoylation, TMX1 is recruited to the MAMs, where it binds and inhibits SERCA2b. As such, loss of TMX1 accelerates SERCA2b-mediated ER Ca2+ accumulation, particularly in the MAMs. As a consequence, loss of TMX1 in HeLa and A375P, a malignant melanoma cell line, increased ER Ca2+ retention and reduced ER–mitochondrial Ca2+ transfer. This led to a reduction in mitochondrial bioenergetics, thereby lowering ATP production and the oxygen consumption rate. Consistent with the work of Foskett and others (67), loss of TMX1 resulted in increased cell death and increased ROS production in vitro (Table 1). However, in vivo, opposite findings were obtained. Furthermore, while loss of TMX1 in these cancer cell lines accelerated tumor growth, TMX1 overexpression had the opposite effect (75). This might be due to the contribution of the microenvironment, including reduced accessibility of oxygen and nutrients, which may contribute to mitochondrial stress. Interestingly, it was shown that although cancer cells lacking TMX1 proliferate slower and display more spontaneous cell death, they are more resistant to mitochondrial stress inducers like rotenone and antimycin (75). Hence, in vivo, cancer cells may experience ongoing mitochondrial stress and/or shortage of nutrients. Under such conditions, cancer cells that have lost TMX1 expression might have a growth advantage over cancer cells with high TMX1 expression. Alternatively, these cells may display increased autophagy, which is beneficial for cancer cell survival under starvation conditions by providing mitochondrial substrates that feed the TCA cycle and sustain nucleotide biosynthesis (102, 104). However, further work is needed to understand these aspects in more detail. In particular, the differences between IP3R inhibition and loss of TMX1, which both impair mitochondrial bioenergetics and result in spontaneous cell death in vitro but lead to an opposite effect in vivo tumor growth experiments (impaired upon IP3R inhibition versus accelerated upon TMX1 loss) require further research.
ER–MITOCENDRIL Ca2+ SIGNALING UNDERLYING CELLULAR SENECECE AND CANCER CELL DEATH THERAPIES

It is important to note that alterations in ER–mitochondrial Ca2+ transfers will not only impact mitochondrial bioenergetics but also cancer cell senescence and sensitivity toward chemotherapeutic drugs (Figure 1). Adequate ER–mitochondrial Ca2+ transfer has been implicated in oncogene-induced and replicative senescence, a condition characterized by a stable proliferation arrest (69, 111). Cancer cells in which IP,R2, the most sensitive isoform to its ligand IP3, or MCU were knocked down could escape cellular senescence (69). Conversely, cancer cells exposed to a continuous supply of cell-permeable IP3 displayed premature senescence. Strikingly, cells undergoing oncogene-induced senescence displayed an increase in basal mitochondrial Ca2+ and IP3-induced mitochondrial Ca2+ accumulation. Cells lacking IP3,R2 or MCU did not display this mitochondrial Ca2+ rise. Mitochondrial Ca2+ induced cellular senescence by causing a partial depolarization of the mitochondrial membrane and an accumulation of mitochondrial ROS. Moreover, cellular senescence could be mimicked by mitochondrial depolarization by the mitochondrial uncoupler FCCP (69). A further detailed discussion on the alterations in mitochondrial homeostasis and the contributing underlying mechanisms in cellular senescence is provided elsewhere (112).

The adequate ER–mitochondrial Ca2+ transfer underlies the cell death-inducing properties of several chemotherapeutic drugs. Recently, extranuclear p53 has emerged as an important molecular link between chemotherapeutic responses and Ca2+ signaling (77, 113). Upon exposure to chemotherapeutic drugs, p53 was shown to accumulate at the ER membranes where it increases SERCA2b activity (Table 1). This resulted in increased [Ca2+]ER, increasing the likelihood of pro-apoptotic Ca2+ transfers to the mitochondria. Cells that lack p53 or that express oncogenic p53 mutations failed to upregulate SERCA2b activity and display ER–mitochondrial Ca2+ transfers and cell death (72). In addition, cells that lack p53 can be sensitized to chemotherapy by overexpressing SERCA or MCU, facilitating ER–mitochondrial Ca2+ transfer (78). Thus, downregulation of ER–mitochondrial Ca2+ fluxes may not only favor cancer cell survival (e.g., by upregulating autophagy) but could also lead to cell-death resistance, as has been shown recently for tumor cells lacking PML (71) or FATE1 (82). FATE1 is a cancer-testis antigen, which localizes at the ER–mitochondrial interface (82). Recently, it has been identified as an MAMs spacer, thereby impairing mitochondrial Ca2+ uptake. As a consequence, FATE1 upregulation, like in adrenocortical carcinoma cells, results in cell-death resistance not only in response to pro-apoptotic stimuli that impinge on ER–mitochondrial Ca2+ signaling but also in response to mitotane, a chemotherapeutic drug clinically used in the treatment of patients with adrenocortical cancer. Moreover, FATE1 expression is also inversely correlated with the overall survival of adrenocortical cancer patients (82). Oppositely, enhancing ER–mitochondrial Ca2+ transfer will favor cell-death therapies (92). Interestingly, some anticancer drugs might actually impact ER–mitochondrial contact sites and thereby enhance the response to other chemotherapeutics. For instance, ABT-737, a non-selective Bcl-2/Bcl-XL inhibitor (114, 115) could reverse the cisplatin resistance in ovarian cancer cells due to increased ER–mitochondrial Ca2+ contact sites (116). Specifically, the authors demonstrated that ABT-737 enriched cisplatin-induced GRP75 and Mfn-2 content at the ER–mitochondria interface. The latter event led to enhanced mitochondrial Ca2+ overload and subsequent cell death (116). Moreover, tumor suppressors at MAMs, including p53, were reported to modulate Ca2+ transfer and the contact sites (54). Another anticancer compound, whose mechanism involves a Ca2+-dependent step is resveratrol (79). This natural compound selectively increased the mitochondrial Ca2+ uptake of cancer cells, while normal cells remained unaffected. Similarly to other phenols, resveratrol inhibits ATP synthase and impairs ATP production, thereby decreasing mitochondrial [ATP] without affecting cytosolic [ATP] (117). This resulted in suppressed SERCA activity, particularly at the MAM interface, thereby increasing the net flux of Ca2+ through IP3Rs and augmenting mitochondrial uptake (Table 1). The striking difference between the mitochondrial Ca2+ uptake in cancer and in normal cells in the presence of resveratrol was attributed to the enhanced and more stable MAMs in cancer cells, which facilitate the ER–mitochondrial Ca2+ transfer (79). In addition to this, resveratrol can induce autophagy via a mechanism that requires cytosolic Ca2+ and the presence of IP3Rs. In this study, resveratrol triggered a depletion of the ER in intact cells independently of IP3Rs, but not in permeabilized cells where Ca2+ stores are loaded by application of ATP, arguing against a direct inhibition of SERCA by resveratrol. Thus, these findings may relate to an in cellulo decline in SERCA activity due to a decline in ATP (118).

CONCLUSION

Endoplasmic reticulum–mitochondrial Ca2+ fluxes impact several cancer hallmarks, including mitochondrial metabolism, autophagy, apoptosis resistance, and metastasis. It is very likely that different tumor stages require different levels of ER–mitochondrial Ca2+ flux for instance to ensure cell survival at early stages, promote invasion at intermediate stages and tumor growth at late stages. Moreover, different oncogenes and tumor suppressors exert their part of their function at the MAMs by impacting Ca2+-transport systems.

An emerging concept is that cancer cells become addicted to constitutive ER–mitochondrial Ca2+ transfers. Thus, suppressing these basal ongoing ER–mitochondrial Ca2+ fluxes represent a therapeutic strategy to target tumor cells thereby suppressing their survival, invasion and growth.

In contrast to this, ER–mitochondrial Ca2+ fluxes appear instrumental for proper therapeutic responses to chemotherapeutic drugs, since an adequate ER–mitochondrial Ca2+ transfer is important for their cell death-inducing properties. Hence, enhancing ER–mitochondrial Ca2+ transfer may provide an attractive strategy to overcome cell death resistance of certain types of cancer toward chemotherapeutics.

Hence, it is expected that both dampening and boosting ER–mitochondrial Ca2+ transfers hold therapeutic potential,
dependent on the clinical stage of the tumor and the applied anticancer strategy. However, a major challenge will be to limit these effects to cancer cells, as obviously ER–mitochondrial Ca\(^{2+}\) fluxes also underlie the survival of healthy cells. Nevertheless, the presence, composition, and properties of ER–mitochondrial contact sites in healthy versus cancer cells and the dependence of these cells on these sites for cell survival may be strikingly different, creating a therapeutic window for the selective targeting of cancer cells while sparing healthy cells.

**AUTHOR CONTRIBUTIONS**

GB and RR drafted the manuscript. HI, MK, RR, and GB wrote parts of the manuscripts. HI, RR, and GB prepared figures. All the authors have read and approved the final version of the manuscript.

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