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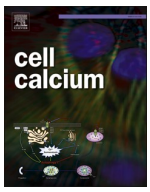

Ca^{2+} signaling and cell death

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ABSTRACT

Multiple forms of regulated cell death (RCD) have been characterized, each of which originates from the activation of a dedicated molecular machinery. RCD can occur in purely physiological settings or upon failing cellular adaptation to stress. Ca^{2+}ions have been shown to physically interact with – and hence regulate – various components of the RCD machinery. Moreover, intracellular Ca^{2+} accumulation can promote organellar dysfunction to degree that can be overtly cytotoxic or sensitize cells to RCD elicited by other stressors. Here, we provide an overview of the main links between $Ca^{2+}$and different forms of RCD, including apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, lysosome-dependent cell death, and parthanatos.

1. Introduction

Different cell death modalities exhibit specific morphological, biochemical, and immunological features, leading to the formulation of guidelines for the definition and comprehension of the cellular demise that are periodically revised and updated [1,2]. The term regulated cell death (RCD) is generally used to define an active cytotoxic program that relies on a specific molecular machinery, and hence can be modulated by genetic or pharmacological interventions [1]. RCD can occur in purely physiological conditions as part of a genetically encoded program for organismal development of homeostasis, and in such case is commonly referred to as programmed cell death (PCD) [3–5]. Alternatively, RCD can emerge upon failing cellular adaptation to detrimental changes of the intracellular or extracellular microenvironment. By eliminating dysfunctional or even potentially oncogenic cells, stress-induced RCD also contributes to the preservation of organismal homeostasis despite compromised cellular fitness [6,7]. Recently, cell death nomenclature has been refocused on quantitative and biochemical over qualitative and morphological aspects of the process, with terms including apoptosis, necroptosis, mitochondrial permeability transition (MPT)-driven necrosis, parthanatos, ferroptosis and pyroptosis referring to precise molecular mechanisms [1].

The first connection between Ca^{2+} signaling (Box 1) and cell death dates back to about 50 years ago, when it was proposed that ischemia-driven myocardial necrosis might be a consequence of excessive Ca^{2+} entry into myocytes [8]. Nowadays, Ca^{2+} ions have been involved in the regulation of a myriad of cellular processes including both the initiation and the execution steps of cell death as elicited by numerous stressors [9,10]. Moreover, intracellular Ca^{2+} overload constitutes a major perturbation of intracellular homeostasis that affects a variety of organelles and potentially culminates with or predisposes to RCD [11–13]. This implies that virtually all RCD variants can be influenced by intracellular Ca^{2+} fluxes (Fig. 1).

Here, we provide an overview on the main links between Ca^{2+} signaling and RCD.

2. Ca^{2+} signaling and apoptotic cell death

Apoptosis is an RCD variant that involves the activation of one or more proteases of the caspase family, notably executioner caspase 3

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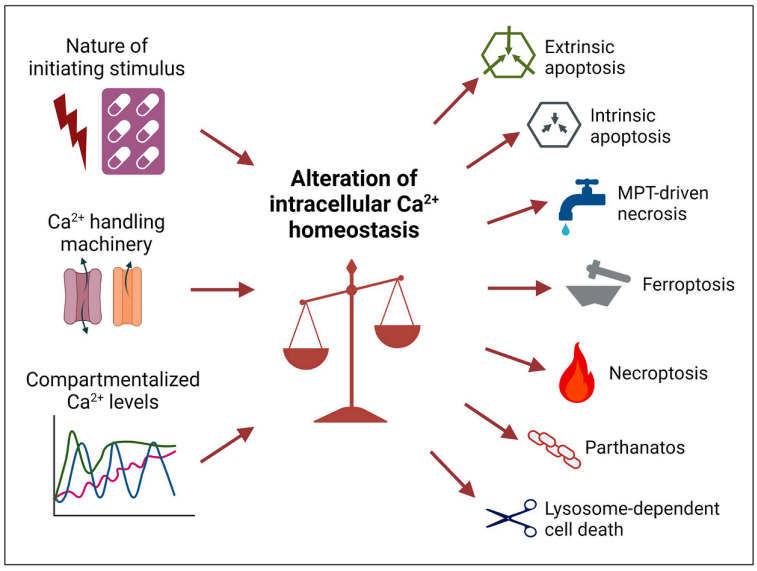

Fig. 1. The connection between Ca^{2+} signaling and cell death modalities. Multiple factors dictate cellular responses to Ca^{2+} fluxes, including (but not limited to): (1) The intensity, nature, and duration of Ca^{2+}-mobilizing stimuli, (2) expression levels of Ca^{2+} transporters, Ca^{2+}-buffering proteins and other components of the machinery for Ca^{2+} handling, and (3) compartmentalized Ca^{2+} kinetics and concentrations. Created with BioRender.com.

(CASP3) [13]. Beside playing a critical role in morphogenesis as a *bona fide* form of PCD, apoptosis participates in the etiology of numerous pathologies originating from the unbalanced loss of terminally differentiated cells, including cardiovascular conditions and neurological disorders [13]. Conversely, defects in apoptotic signaling have been consistently linked to malignant transformation and tumor progression [14,15]. At least in some experimental models, inhibiting Ca^{2+} efflux from the endoplasmic reticulum (ER) by blocking 1,4,5-trisphosphate receptors (IP_3Rs) reportedly protects cells from apoptosis as induced by a variety of stimuli [16–18]. Most often, though, the impact of Ca^{2+}fluxes on apoptotic signaling has been investigated in more defined experimental systems.

So-called "extrinsic apoptosis" is generally initiated by the interaction of dedicated plasma receptors known as "death receptors" (DRs), such as Fas cell surface death receptor (FAS, also known as CD95), with their cognate ligands [19]. This results in the assembly of a supramolecular complex at the intracellular tail of DRs that promotes the sequential activation of CASP8 and CASP3, in some cases upon mitochondrial involvement and consequent release of cytochrome c, somatic (CYCS) [20]. FAS-driven apoptosis has been consistently associated with cytosolic Ca^{2+} elevations [21–23]. More specifically, FAS activation has been linked to a biphasic alteration in cytosolic Ca^{2+}levels: (1) a rapid Ca^{2+} discharge from the ER driven by the phospholipase C (PLC)-dependent production of the inositol IP_3R agonist inositol 1,4,5-trisphosphate (IP_3) [22,24]; followed by (2) a delayed, sustained increase in cytosolic Ca^{2+}abundance [22,24]. However, while some authors suggest the latter to originate from the ER in response to the cytosolic accumulation of CYCS and promote apoptosis [22], others attribute delayed cytosolic Ca^{2+}waves elicited by DRs to extracellular stores under the control of ORAI calcium release-activated calcium modulator 1 (ORAI1) and stromal interaction molecule 1 (STIM1) and propose an anti-apoptotic role for them downstream of protein kinase C beta (PRKCB) activation [24,25]. These findings suggest that cytosolic Ca^{2+} elevations may have opposite effects on extrinsic apoptosis, potentially reflecting (1) the precise nature of the initiating stimulus, (2) expression levels of key components of the Ca^{2+} handling and/or apoptotic machinery; and (3) compartmentalized Ca^{2+} levels. In line with this notion, extracellular Ca^{2+} influx via transient receptor potential (TRP) channels sustains (rather than inhibits) apoptosis driven by tumor necrosis factor (TNF) in hepatocellular carcinoma cells, with Ca^{2+}-mobilizing agents exacerbating TNF cytotoxic effects in the absence of mitochondrial involvement [26]. Conversely, TNF exerts neuroprotective effects against glutamate challenges, which are also associated with alterations of Ca^{2+} influx [27].

At odds with its extrinsic counterpart, intrinsic apoptosis is initiated by perturbations of the intracellular microenvironment (e.g., DNA damage) [28] and is demarcated by irreversible mitochondrial outer membrane permeabilization (MOMP) under control by pro- and anti-apoptotic members of the BCL2 apoptosis regulator (BCL2) protein family [13,29,30], followed by CASP3 activation [31–33]. MOMP as mediated by pore-forming BCL2 family members including BCL2 associated X, apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1) is tightly linked to Ca^{2+} release from the ER via IP_3Rs and consequent uptake by mitochondria [34–36]. Interestingly, BCL2 family apoptosis regulator BOK (BOK) not only can induce MOMP independent of BAX and BAK1 [37,38], but also resides at the ER [39], where it forms a complex with IP_3Rs that preserves them from degradation [40,41]. That said, whether the pro-apoptotic functions of BOK involves alterations of ER Ca^{2+} efflux via IP_3Rs remains to be fully elucidated [40,42–44]. In line with this notion, anti-apoptotic BCL2 family members including BCL2 itself and BCL2 like 1 (BCL2L1; best known as BCL-X_L) robustly limit the transfer of Ca^{2+} ions from the ER to mitochondria, not only by lowering baseline ER Ca^{2+} levels [45–47], but also by (1) inhibiting IP_3R opening [48–50], and (2) limiting mitochondrial Ca^{2+} accumulation via voltage-dependent anion channel 1 (VDAC1) [51,52]. Whether the latter mechanism is operation in all cells or whether at least in some instances anti-apoptotic BCL2 proteins instead promote mitochondria Ca^{2+}uptake via VDAC1, however, remains debated [53,54].

Importantly, various other oncogenic proteins and tumor suppressors share the ability to modulate the transfer of Ca^{2+}ions from the mitochondria to the ER [35,55–57] or control the activity of mitochondrial Ca^{2+} channels [58,59], resulting in altered RCD susceptibility. In line with this notion, genetic manipulation of major ER-mitochondria Ca^{2+} transfer effectors including the IP_3R, VDACs and the mitochondrial calcium uniporter (MCU) complex has been shown to change the propensity of mammalian cells to undergo RCD in response to treatments that elicit cytosolic Ca^{2+}accumulation, including numerous chemotherapeutics [60–63]. That said (at least in some cellular settings), a

sustained transfer of Ca^{2+} ions from the ER to mitochondria does not culminate with RCD, but instead promotes the an irreversible proliferative arrest (i.e., cellular senescence) as a consequence of a Ca^{2+}-dependent remodeling of mitochondrial metabolism [64–66]. In this context, pharmacological BCL-X_L inhibitors may act as senolytic agents not only because of their capacity to drive RCD in senescent cells [67,68], but also owing to their ability to concomitantly block the transfer of Ca^{2+} ions from the ER to mitochondria, which is supported by BCL-X_L when expressed at low levels [69]. These observations also suggest that (1) cells with a high energetic demand (i.e., malignant cells) may intrinsically suffer from limitations in mitochondrial Ca^{2+} uptake [70] and (2) IP_3R-mediated Ca^{2+} oscillations might increase cellular resistance to RCD-promoting insults.

Of note, mitochondrial permeabilization has also been suggested as a major cytotoxic effector of cytosolic Ca^{2+}waves as elicited by the opening of plasma membrane channels such as transient receptor potential cation channel subfamily V member 1 (TRPV1) [71,72] and ORAI1/STIM1 [73]. However, most of these studies fail to mechanistically address the implication of MOMP by genetic approaches, implying – that at least potentially – other RCD mechanisms may be involved (see below). Accordingly, BAX and BAK1 have been involved in RCD modalities other than apoptosis [74,75].

Taken together, these observations exemplify the key role of cytosolic Ca^{2+}as both an inducer and a regulator of apoptotic RCD.

3. Ca^{2+} signaling and other forms of regulated cell death

3.1. MPT-driven necrosis

Mitochondrial permeability transition (MPT)-mediated is a form of RCD driven by a sudden increase in the permeability of the inner mitochondrial membrane (IMM) to small solutes, leading to rapid mitochondrial transmembrane potential ($\Delta\psi_m$) dissipation and consequent osmotic breakdown of the organelle [76]. The process is initiated by the opening of a hitherto poorly characterized multiprotein channel commonly known as permeability transition pore complex (PTPC) and is negatively regulated by peptidylprolyl isomerase F (PPIF, best known as CYPD) [76]. At least in some circumstances, mitochondrial Ca^{2+} overload coupled to reactive oxygen species (ROS) overproduction [77] induces increases PTPC conductance and hence drives MPT [78]. In line with this notion, various interventions affecting mitochondrial Ca^{2+} entry including manipulations of the MCU complex alter PTPC threshold for opening, hence affecting cellular sensitivity to MPT. Thus, deletion of *Mcu* – which encodes the pore-forming subunit of the MCU complex – has been shown to inhibit PTPC opening in both neurons and cardiomyocytes, at least in mice [79,80]. Conversely, interfering with the correct assembly of the MCU [81], deleting *Mcub* (which encoded an MCU paralog) [82], as well as depleting mitochondrial calcium uptake 1 (MICU1) [83] reportedly increase mitochondrial Ca^{2+} uptake and lower PTPC threshold, resulting in cellular sensitization to MPT. Nevertheless, blocking the primary mechanism of mitochondrial Ca^{2+} efflux in the heart by deletion of *Slc8b1* imposed aberrant ROS production and necrotic death due to mitochondrial Ca^{2+} overload [84].

Of note, whole-body *Mcu* deletion as well as the cardiomyocyte-specific overexpression of a dominant-negative MCU variant does not mediate cardioprotective effects despite reduced mitochondrial Ca^{2+} levels [85,86]. This is probably due to the onset of compensatory mechanisms that preserve PTPC sensitivity to Ca^{2+}, including CYPD phosphorylation or increased CYPD binding to the F_OF_1 ATP synthase [87], which plays a prominent role in PTPC formation [88–90]. Interestingly, MCU and the F_OF_1 ATP synthase appear to form a mega-complex in various eukaryotes including human cells [91], but whether this interaction regulates MPT-driven necrosis remains to be elucidated.

Recently, the PLC activator 2,4,6-trimethyl-N-(m-3-trifluoromethyl-phenyl) benzenesulfonamide (m-3M3FBS) has been shown to evoke robust Ca^{2+} efflux from the ER coupled to mitochondrial Ca^{2+}uptake, mitochondrial permeabilization and RCD in a BAX/BAK1-independent manner, suggesting the involvement of the MPT [92]. In line with this notion, such a process could be blocked with the CYPD inhibitor cyclosporine A (CsA) [92]. However, *Ppif* deletion had no impact on RCD as driven by m-3M3FBS [92], pointing to (1) a novel CYPD-independent activity of CsA, and (2) the existence of a mechanism of mitochondrial permeabilization other than MOMP and MPT [93].

3.2. Necroptosis

Necroptosis is a form of caspase-independent RCD that involves the activation of the pore-forming protein mixed lineage kinase domain like pseudokinase (MLKL), which is generally mediated by receptor interacting serine/threonine kinase 3 (RIPK3) [94,95]. Importantly, altered Ca^{2+} homeostasis appears to be involved in multiple forms of necroptosis [96]. For instance, necroptosis as driven by TNF receptor superfamily member 1A (TNFRSF1A) signaling the context of caspase inhibition results in the MLKL-dependent activation of transient receptor potential cation channel subfamily M member 7 (TRPM7) and consequent Ca^{2+} influx [97]. Moreover, cytosolic Ca^{2+} accumulation as a consequence of ER stress [98] has been shown to drive necroptotic cell death in human leukemia cells exposed to an antimicrobial peptide [99]. Similarly, cardiomyocytes treated with sorafenib (a tyrosine kinase inhibitor currently used for cancer therapy) exhibit increased ER-mitochondria juxtaposition coupled to mitochondrial Ca^{2+} overload, RIPK3 and MLKL activation, and hence active necroptotic signaling [100,101]. In line with this notion, overexpression of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps results in necroptosis inhibition upon improved Ca^{2+} storage in the ER and limited MCU-mediated mitochondrial Ca^{2+} entry [102]. MCU functions also appear to be potentiated by interactions with RIPK1 [103], pointing to a necroptosis-independent role of RIPK1 in the regulation of Ca^{2+} fluxes. Finally, RIPK3 overexpression in cardiomyocytes has been shown to promote cytosolic Ca^{2+} overload upon the establishment of ER stress, culminating necroptotic RCD [104]. Intriguingly, this cytotoxic effect could be at least partially rescued with CsA [104], suggesting the existence of functional interactions between the molecular machineries for MPT and necroptosis.

3.3. Ferroptosis

Ferroptosis is an iron-dependent form of RCD involving abundant ROS generation and consequent peroxidative damage to lipids and other macromolecules [105,106]. In line with this notion, various antioxidant enzymes such as glutathione peroxidase 4 (GPX4), S100 calcium binding protein A4 (S100A4, best known as FSP1) and GTP cyclohydrolase 1 (GCH1) effectively limit lipid peroxidation, thus preventing ferroptosis [105,106]. A prolonged rise in cytosolic Ca^{2+} preceding complete cell rupture has been documented in multiple instances of ferroptosis [107]. Moreover, reducing reticular Ca^{2+} levels evokes a lipid remodeling that protects cells from ferroptosis [108]. Of note, the MCU regulator MICU1 appears to be required for lipid peroxidation during cold stress-induced ferroptosis [109], suggesting that mitochondrial Ca^{2+} uptake may also be involved in ferroptotic RCD. The underlying molecular mechanisms, however, remain to be fully understood.

3.4. Lysosome-dependent cell death

Lysosome-dependent cell death is a form of RCD demarcated by primary lysosomal membrane permeabilization (LMP) and consequent spillage of lysosomal contents to the cytosol [1,110,111]. LMP can be driven by ROS overgeneration upon activation of lysosomal Ca^{2+} efflux channels [112]. Of note, lysosomal Ca^{2+} levels [113] resemble their reticular counterparts, playing a key role in the lysosomal functions. While the mechanisms through which lysosomal Ca^{2+} signaling influences lysosome-dependent cell death remain unclear, transmembrane

Box 1
Fundamentals of Ca^{2+} signaling

Cytosolic Ca^{2+} levels are tightly regulated, reflecting the central role of cytosolic Ca^{2+} as a second messenger in multiple signaling pathways. Specifically, cytosolic Ca^{2+} levels are low in resting cellular conditions (~100 nmol/L), as compared to the both extracellular environment (~2 mmol/L) and the lumen of intracellular Ca^{2+} stores such as the endoplasmic reticulum (ER; 0.3–0.7 mmol/L), lysosomes (0.4–0.6 mmol/L) and Golgi apparatus (GA; 0.2–0.5 mmol/L) [121,122]. Such a tight control is ensured by complex toolkit involving channels, pumps, exchangers, transporters, and Ca^{2+} binding proteins that are expressed at virtually all cellular compartments [123]. In response to a variety of stimuli, including extracellular transmitters and hormones, cytosolic Ca^{2+} levels increase (i) via Ca^{2+} influx from the extracellular milieu, which is mediated by plasma membrane channels including members of the transient receptor potential (TRP) and voltage-gated Ca^{2+} (VGC) channel superfamilies [124,125]; or (ii) via Ca^{2+} release from internal stores, mostly the ER and GA, upon activation of inositol 1,4,5-trisphosphate (IP_3) receptors, lysosomes, upon activation of mucolipin TRP cation channel 1 (MCOLN1, best known as MCOLN1) and two pore segment channel 2 (TPCN2), and the sarcoplasmic reticulum (in myocytes), upon activation of ryanodine receptors [126].

The type of stimulus, its intensity and duration, as well as the presence of Ca^{2+} buffering proteins and mitochondrial status determine cytosolic Ca^{2+} levels achieved upon cellular stimulation. Cytosolic Ca^{2+} levels are detected by dedicated sensors and ultimately translated into specific cellular responses. Free cytosolic Ca^{2+} is rapidly captured by sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps, which transfer Ca^{2+} into the ER lumen, ATPase secretory pathway Ca2+ transporting 1 (ATP2C1, best known as SPCA), which pumps Ca^{2+} into the GA, and (to a smaller extent) lysosomes, which appear to accumulate Ca^{2+} through an H^+-dependent mechanism [127]. Alongside, Ca^{2+} signaling is switched off by extrusion through ATPase plasma membrane Ca2+ transporting 1 (ATP2B1, best known as PMCA) pump or solute carrier family 8 member A1 (SLC8A1, best known as NCX1) [128]. Of note, a slow, capacitive Ca^{2+} influx from the extracellular space is necessary to refill intracellular stores. This is because PMCA works faster than refilling mechanisms, ultimately generating a process termed store-operated Ca^{2+} entry (SOCE) [71]. Thus, upon ER Ca^{2+} depletion, ER-resident members of the stromal interaction molecule (STIM) protein family oligomerize and redistribute to ER-plasma membrane junctions, where they recruit and activate ORAI calcium release-activated calcium modulator (ORAI). This enables the assembly of the core component of Ca^{2+} release-activated Ca^{2+} (CRAC) channels, which mediates Ca^{2+} influx.

While mitochondria and peroxisomes limit cytosolic Ca^{2+} elevations by sequestering high amounts of Ca^{2+}ions, they are not considered constitutive Ca^{2+} stores. In resting conditions, mitochondrial Ca^{2+} levels resemble their cytosolic counterparts, but upon stimulation they can increase up to 10-fold. This is mainly due to the existence of an electric potential across the inner mitochondrial membrane (IMM), and the very close apposition of mitochondria to major intracellular Ca^{2+} sources, particularly the ER [129]. In this setting, Ca^{2+} ions readily cross the outer mitochondrial membrane (OMM) through voltage-dependent anion channels (VDACs), ultimately accumulating inside the matrix via the mitochondrial calcium uniporter (MCU) complex. Mitochondrial Ca^{2+} signaling is terminated by the activity of two Ca^{2+} efflux mechanisms, a Na+/Ca^{2+} exchanger encoded by *SLC8B1* and a hitherto poorly identified H^+/Ca^{2+} antiporter.

BAX inhibitor motif containing 1 (TMBIM1) has recently been shown to increase lysosomal Ca^{2+} content and thus promote lysosomal acidification, resulting in primary LMP and cell death during lysosomal stress [114].

3.5. Parthanatos

The term parthanatos refers to a caspase-independent type of RCD marked by poly(ADP-ribose) polymerase-1 (PARP1) hyperactivation and consequent translocation of apoptosis inducing factor mitochondria associated 1 (AIFM1) from mitochondria to the nucleus [106,115]. Similar to other RCD modalities, parthanatos has also been linked to altered Ca^{2+} fluxes. Thus, increased cytosolic Ca^{2+} levels have been associated with neuronal death as initiated by PARP1 activation [116]. Moreover, glutamate has been shown to trigger parthanatos upon activation of N-methyl-D-aspartate (NMDA) receptors, coupled to massive Ca^{2+} influx [117]. In response to N-methyl-N′-nitro-N′-nitrosoguanidine (MNNG) administration and ischemia/reperfusion, parthanatos reportedly emerged from ER Ca^{2+} spillage in concert with ROS overproduction [118]. However, additional work is required to clarify the mechanistic role of cytosolic Ca^{2+} in parthanatos.

4. Conclusions

Although alterations of Ca^{2+} signaling seem to participate in virtually all forms of cell death, the mechanistic contribution of Ca^{2+} fluxes to RCD remains to be formally assessed in multiple settings, especially (but not only) for recently described RCD variants. Specifically, whether Ca^{2+} fluxes are an active determinant of any steps of RCD as opposed to a passive phenomenon that accompanies (but does not affect) RCD has frequently been overlooked. Mechanistic experiments based on chelators of cytosolic and/or extracellular Ca^{2+} ions, as well as genetic perturbations of Ca^{2+}buffering or transit at specific organelles stand out as critical approaches to precisely dissect the role of Ca^{2+} in RCD. Alongside, it will be important to understand whether specific components of the cellular Ca^{2+} handling machinery intersect with RCD signaling because of their role in Ca^{2+}homeostasis or via other Ca^{2+}-independent effects. Vice versa, it will be interesting to clarify whether components of the molecular machinery for RCD influence Ca^{2+}homeostasis, and whether such an effect is linked to RCD signaling or not [119,120]. In conclusion, additional work is needed to fully elucidate the physical and functional interaction between Ca^{2+}homeostasis and RCD.

CRediT author statement

Nada Dhaouadi: Writing - Original draft preparation; Figures preparation. Veronica Angela Maria Vitto: Writing- Original draft preparation. Paolo Pinton: Writing- Reviewing and Editing. Lorenzo Galluzzi: Writing- Reviewing and Editing, Supervision. Saverio Marchi: Writing- Reviewing and Editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: LG is/has been holding research contracts with Lytix Biopharma, Promontory and Onxeo, has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, The Longevity Labs, Inzen, Imvax, Sotio, Promontory, Noxopharm, EduCom, and the Luke Heller TECPR2 Foundation, and holds Promontory stock options. All other authors have no conflicts to disclose.

Data availability

No data was used for the research described in the article.

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