Biol Res 37: 653-660, 2004

Mitochondrial Ca^{2+} homeostasis in health and disease

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ABSTRACT

Although it has long been known that mitochondria possess a complex molecular repertoire for accumulating and releasing Ca^{2+}, only in recent years has a large body of data demonstrated that these organelles promptly respond to Ca^{2+}-mediated cell stimulations. In this contribution, we will review the principles of mitochondrial Ca^{2+} homeostasis and its signaling role in different physiological and pathological conditions.

Key words: Mitochondria, Calcium, Apoptosis, Bcl-2, viral proteins

INTRODUCTION

The contribution of mitochondria to intracellular Ca^{2+} signaling and the role of mitochondrial Ca^{2+} uptake, both in shaping the cytoplasmic response and controlling mitochondrial function, are currently areas of intense investigation (Rizzuto et al., 2004; Rizzuto et al., 2000; Kroemer and Reed, 2000).

These studies rely to a large extent on the appropriate use of emerging techniques, coupled with judicious data interpretation. Mitochondrial calcium traffic takes place essentially through two pathways: i) an electrophoretic uniporter, allowing the accumulation of Ca^{2+} down its electrochemical gradient (~ 180 mV, negative inside), and ii) an electroneutral antiporter that by exchanging Ca^{2+} with either Na^+ or H^+ prevents the attainment of an electrochemical equilibrium. (Bernardi, 1999; Duchen, 2000). Based on these considerations, and on the extensive experimental work carried out in the 1960s and 1970s, mitochondria were considered important components of the Ca^{2+} handling machinery of the cell, that could accumulate and release the cation in physiological conditions (Crompton et al., 1978; Bragadin et al., 1979). This concept was almost completely reversed in the following decade, when seminal work by Berridge and coworkers demonstrated that the increases in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) elicited by different extracellular stimuli occurred through the production of inositol 1,4,5 trisphosphate that induces the opening of an intracellular channel, located in the endoplasmic reticulum (ER).

In the following years, biochemical and molecular studies identified the molecules (the IP_3-gated channels, the Ca^{2+} ATPase the lumenal Ca^{2+} handling proteins) through which the ER exerts the role of agonist-sensitive intracellular Ca^{2+} store (Pozzan et al., 1994; Berridge et al., 2000; Berridge et al., 2003).

Finally, it became apparent that isolated mitochondria placed in a buffered medium containing Ca^{2+} at a concentration similar to that transiently observed in the cytosol upon histamine stimulation (1–2 μM), do not show significant increases in matrix $[Ca^{2+}]$ (Rizzuto et al., 1993).

Thus, the general consensus became that mitochondria were suited for Ca^{2+} uptake during Ca^{2+} overload, and thus their role was considered to be significant in

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Received:

pathologies in which Ca^{2+} dysregulation could occur, e.g. during neuronal excitotoxicity (Nicotera and Orrenius, 1992).This scenario has been reversed since it became possible to directly measure $[Ca^{2+}]_m$ using organelle specific probes (such as the photoprotein aequorin, the positively charged fluorescent dyes accumulated in the mitochondria and, more recently, the recombinant fluorescent indicators obtained by molecularly engineering GFP) (Chiesa et al., 2001; Zhang et al., 2002). In this way several laboratories showed that rapid increases of $[Ca^{2+}]_m$ that are much larger than those of $[Ca^{2+}]_c$ occurred during agonist stimulation (Rizzuto et al., 1993; Hajnoczky G et al., 1995). These results were surprising, given the low affinity of the mitochondrial Ca^{2+} uptake systems. However, such an apparent contradiction has been explained showing that ER and mitochondria have a close structural relationship. Mitochondria are thus capable of sensing microdomains of high $[Ca^{2+}]$ generated in close proximity to ER Ca^{2+} release channels (Rizzuto et al., 1998), that are sufficient to cause accumulation through the low-affinity transporters of the inner membrane, the "uniporter," recently shown to be a selective Ca^{2+} channel (Fig.1).

MITOCHONDRIA PARTICIPATE IN PHYSIOLOGICAL Ca^{2+} SIGNALING

Mitochondrial participation in physiological Ca^{2+} signaling soon became a widely accepted notion, and work was initiated to clarify its role. A first potential function could be inferred by the fine biochemical work carried out by Denton, McCormack and Hansford (Hansford and Chappell, 1967; Denton et al., 1980; McCormack et al.,

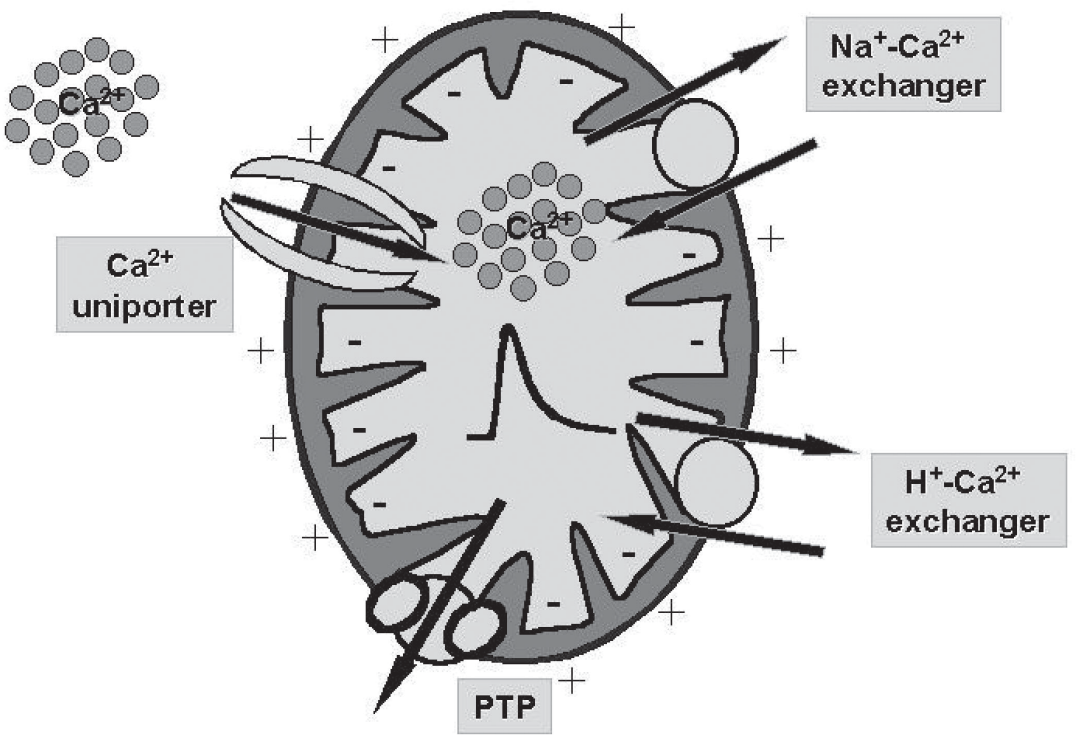

Figure 1. Mitochondrial Ca2+ transport pathways in energized mitochondria. The figure shows the principle players of mitochondrial Ca^{2+} homeostasis. Ca^{2+} enters mitochondria through an electrogenic pathway, the "Ca^{2+} uniporter," recently shown to be a selective channel (Kirichok et al., 2004). The Ca^{2+} efflux pathways are also schematically shown: the H^+-Ca^{2+} and Na^+-Ca^{2+} exchangers, as well as the permeability transition pore (PTP).

1990; McCormack and Denton, 1990) who demonstrated that in the matrix, Ca^{2+} stimulates the activity of three Ca^{2+}-sensitive dehydrogenases of the Krebs cycle (NAD^+-isocitrate-, 2-oxoglutarate-, and pyruvate-dehydrogenase) thus enhancing the electron flow through the electron transport chain and increasing ATP production (Jouaville et al., 1999). Thus, an obvious function for mitochondrial Ca^{2+} homeostasis could be found, i.e. that of rapidly adapting aerobic metabolism to the increased needs of a stimulated cell. However the following work revealed a much broader picture, demonstrating not only that mitochondrial Ca^{2+} uptake influences the kinetics and spatial properties of the $[Ca^{2+}]_c$ rise in the cytoplasm, but also that within the mitochondria a Ca^{2+}-mediated signal can induce a radically different effect, i.e. the triggering of apoptosis. Indeed, Jouaville and co-workers showed that the pattern of InsP3-dependent Ca^{2+} waves of Xenopus laevis oocytes is modulated by Ca^{2+} uptake by energized mitochondria (Jouaville et al., 1995), and several groups demonstrated the role of mitochondria in buffering cytosolic $[Ca^{2+}]$ changes in neurons, (Werth and Thayer, 1994; Kiedrowski and Costa, 1995; Budd and Nicholls, 1996) adrenal chromaffin (Herrington et al., 1996) and smooth muscle cells (Drummond and Fay, 1996).

Moreover, mitochondrial Ca^{2+} uptake was then shown to modulate both Ca^{2+} release by the IP3-sensitive channel of the ER (Hajnoczky et al., 1995) and Ca^{2+} influx through CRAC channel of the plasma membrane (Hoth et al., 1997; Gilabert and Parekh, 2000). Thus, mitochondria acting as fixed buffers that can finely tune the spatio-temporal patterns of cellular Ca^{2+} signals and their subcellular distribution, the regulation of which is still largely unknown, may greatly influence the final outcome of a Ca^{2+}-mediated physiological or pathological challenge. More recently, it was proposed that in neurons mitochondria act as a rapidly mobilisable Ca^{2+} pool, that releases Ca^{2+} at the neuromuscular junction and is responsible for post-tetanic potentiation of neurotransmitter release (Rizzuto, 2003). This would already represent a wide repertoire of cellular consequences for mitochondrial Ca^{2+} uptake. In the second part of the review, we will focus on the other intramitochondrial effect, i.e. that of triggering large-scale alterations of organelle morphology, and hence the release of proteins normally located within the mitochondria (e.g. cytochrome c, Smac/DIABLO, AIF) into the cytoplasm, where they act as caspase cofactors (Hengartner, 2000). In other words, recent work by us and other groups highlighted the role of Ca^{2+} signals reaching the mitochondria in the activation of apoptosis.

MITOCHONDRIAL Ca^{2+} SIGNALS HAVE A ROLE IN APOPTOSIS

Studying the oncoprotein Bcl-2 and its mechanism of action has established a critical link between Ca^{2+} and apoptosis. The Bcl-2 (B-cell lymphoma/leukaemia-2) family of proteins plays a major role in the control of apoptosis and displays either pro- or anti-apoptotic functions. They contain at least one of the four conserved regions called Bcl-2 homology domain (BH1-BH4). These motifs are formed by α-helices and enable the different members of the family to form either homo or heterodimers. The members that inhibit apoptosis, such as Bcl-2, Bcl-xL harbor at least three BH domains. Among the death promoters, some proteins, e.g. Bax, contain BH1, BH2 and BH3, and closely resemble Bcl-2. Others (e.g. Bid, Bad) posses only the BH3 domain, which is essential for both their interaction with other family members and their death promoting activity. Most Bcl-2 family of proteins also contain a C-terminal 20-residue hydrophobic domain (the putative transmembrane domain, TM) that targets them to intracellular membranes. In the case of Bcl-2, the TM functions as a signal anchor that targets and inserts the protein into the two main membrane locations for this protein: the mitochondrial outer membrane and the endoplasmic/nuclear envelope. In the case of Bax, the TM domain permits its translocation from cytosol to the mitochondrial membranes upon overexpression and/or induction of

cell death (Chao and Korsmeyer, 1998; Droin and Green, 2004).

It has been shown that Bcl-2 can act as an ion channel in isolated lipid bilayers (Schendel et al., 1997). Thus, Bcl-2 could alter ion homeostasis of the intracellular organelles where it putatively localizes (Minn et al., 1997). Thus we investigated its effect on calcium signaling. To address this issue, we recombinantly expressed Bcl-2 together with the targeted chimeras of the Ca^{2+}-sensitive photoprotein aequorin, that are specific Ca^{2+} probes for the different subcellular compartments (the ER, the mitochondria, the cytoplasm). In transiently transfected HeLa cells, we observed that Bcl-2 overexpression reduced by about 30% the state of filling of intracellular Ca^{2+} stores (ER and Golgi apparatus) and consequently the stimulus-dependent $[Ca^{2+}]$ increases in the cytoplasm and in mitochondria. The decrease of the steady state $[Ca^{2+}]_{er}$ was not due to a lower rate of Ca^{2+} accumulation, but rather to an increased passive Ca^{2+} leak from the organelle, thus excluding an effect on the pumping activity of the SERCA and pointing to an enhanced channel activity (due either to Bcl-2 itself, or to the stimulation of a yet-unidentified "leak channel") (Pinton et al., 2000). We then investigated whether this alteration in Ca^{2+} homeostasis played a role in the anti-apoptotic activity of Bcl-2. To this end, we replicated the reduction of $[Ca^{2+}]_{er}$ through different experimental maneuvers (and thus independently of Bcl-2, excluding other activities of the oncoprotein). Specifically, we used low doses of tBuBHQ, an inhibitor of the ER Ca^{2+} ATPase (SERCA), co-transfection of the plasma membrane Ca^{2+} ATPase, and incubation in a medium with sub-physiological $[Ca^{2+}]$: in all cases we could observe that a partial reduction of $[Ca^{2+}]_{er}$ corresponds to a reduced sensitivity to an apoptotic agent (ceramide) that is known to act through a "mitochondrial pathway" in a Bcl-2 sensitive manner. Conversely, overexpression of the SERCA led to an increase in both $[Ca^{2+}]_{er}$ levels and in the apoptotic efficacy of ceramide (Pinton et al., 2001). As to the target of the "apoptotic" Ca^{2+} signals, the experimental evidence obtained points to mitochondria. Indeed, we could observe that ceramide treatment (that induces Ca^{2+} release from the ER and Ca^{2+} uptake by mitochondria) induces a major alteration of organelle morphology that is prevented by all experimental conditions that reduce $[Ca^{2+}]_{er}$, and thus the Ca^{2+} signals evoked by physiological and pathological stimuli. As to the mechanism of this morphological alteration (fragmentation, swelling), that allows the release of the caspase co-factors, a key role is supposed to be played by the permeability transition pore (PTP), a high-conductance non-selective channel, the opening/closing characteristics of which are influenced by multiple parameters (e.g. matrix pH, the redox state of critical matrix thiols, the $NAD(P)H_2$/NAD(P) and the concentration of Ca^{2+} in the mitochondrial matrix) (Bernardi, 1999) (Kroemer, 2003). It can be envisioned that coincidence detection of the $[Ca^{2+}]_m$ rise and other pro-apoptotic signals (e.g. oxidative stress or a direct action of the lipid mediator) determines the apoptotic recruitment of mitochondria, and thus the translation of a Ca^{2+} signal into cell death (Ferrari et al., 2002). Interestingly, the pro-apoptotic members of the Bcl-2 family, Bax and Bak, were more recently shown to affect Ca^{2+} homeostasis in exactly the opposite way: double knockouts of Bax and Bak have a reduced $[Ca^{2+}]_{er}$, indicating that they counteract the effect of Bcl-2 on Ca^{2+} homeostasis (indeed, Bcl-2 silencing in the Bax/Bak -/- cells restores the $[Ca^{2+}]_{er}$ levels to values comparable to wild type cells, and increased susceptibility to apoptotic agents) (Rizzuto et al., 2003).

Recently, by investigating the effect on Ca^{2+} homeostasis of a pro-apoptotic protein of Hepatitis B (HBx) we obtained an interesting confirmation to this scenario. HBx is a multifunctional protein, which was proposed to act on different targets (transcription factors, mitochondrial proteins, cytoplasmic kinases, and possibly also Ca^{2+} homeostasis). Also in this case, we employed the aequorin chimeras to investigate, in co-transfection experiments, the effect of HBx on the Ca^{2+} handling of the different cell compartments. Interestingly, the pro-apoptotic protein augments the cytosolic Ca^{2+} signals evoked

by $InsP_3$-linked agonists. However, this is not due to ER Ca^{2+} overload, as the steady state $[Ca^{2+}]_{er}$ levels were identical to those of mock-transfected cells. In the search for the mechanism, we investigated the possibility that, similar to that reported in staurosporin-treated neurons, the Ca^{2+} extrusion capacity of the cell was impaired by a caspase-3 dependent cleavage of the plasma membrane Ca^{2+} ATPase (Schwab et al., 2002). This proved to be the case: in HBx-transfected cells we could detect caspase-3 activation and PMCA cleavage, and recombinant expression of a non-cleavable PMCA mutant both restored the Ca^{2+} signaling patterns and amplitude to those of control cells and reduced the apoptotic efficiency of the viral protein. PMCA is a very effective target for enhancing Ca^{2+} responses, because it represents the most powerful route, allowing the rapid return of $[Ca^{2+}]_c$ to basal level. Also in this case, mitochondria appear to be the target of the "apoptotic" Ca^{2+} signal, and the PTP the most likely molecular mechanism (cyclosporin A, a blocker of the PTP, greatly reduces the apoptotic efficiency of HBx) (Chami et al., 2003) (Fig.2).

A variety of pro- and anti-apoptotic proteins and mediators thus appear to converge on Ca^{2+} signaling, with conceptually different molecular mechanisms: the modulation of ER Ca^{2+} levels (Bcl-2, Bax) the synergistic activity on the mitochondrial effector (ceramide), the impairment of the molecular routes for terminating the $[Ca^{2+}]_c$ rise (HBx) (Pinton et al., 2001; Scorrano et al., 2003). In all cases, the final result is that of shifting the intramitochondrial decoding between the physiological activation of aerobic metabolism (needed in a healthy, stimulated cell) and the release of caspase cofactor (in a cell doomed to apoptotic death) (Fig.3).

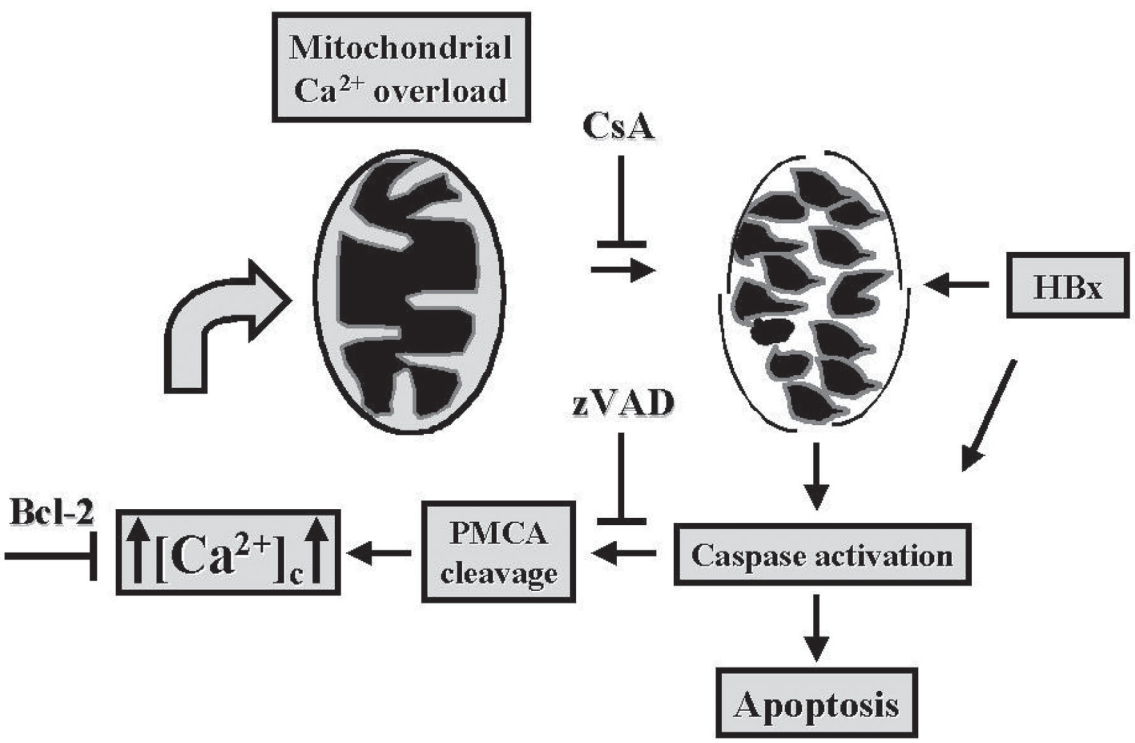

Figure 2. HBx effect on Ca^{2+} signaling and its role in HBx-induced apoptosis. HBx is able to induce apoptosis through mitochondrial dysfunction and caspase activation as shown in the right side of the figure. The larger $[Ca^{2+}]_c$ transients due to PMCA cleavage by the caspases (left side of the figure) alters mitochondrial structure and cause further release of caspase co-factors, which definitively take the cell to death (affirming the basic role of Ca^{2+} in the apoptotic procedure).

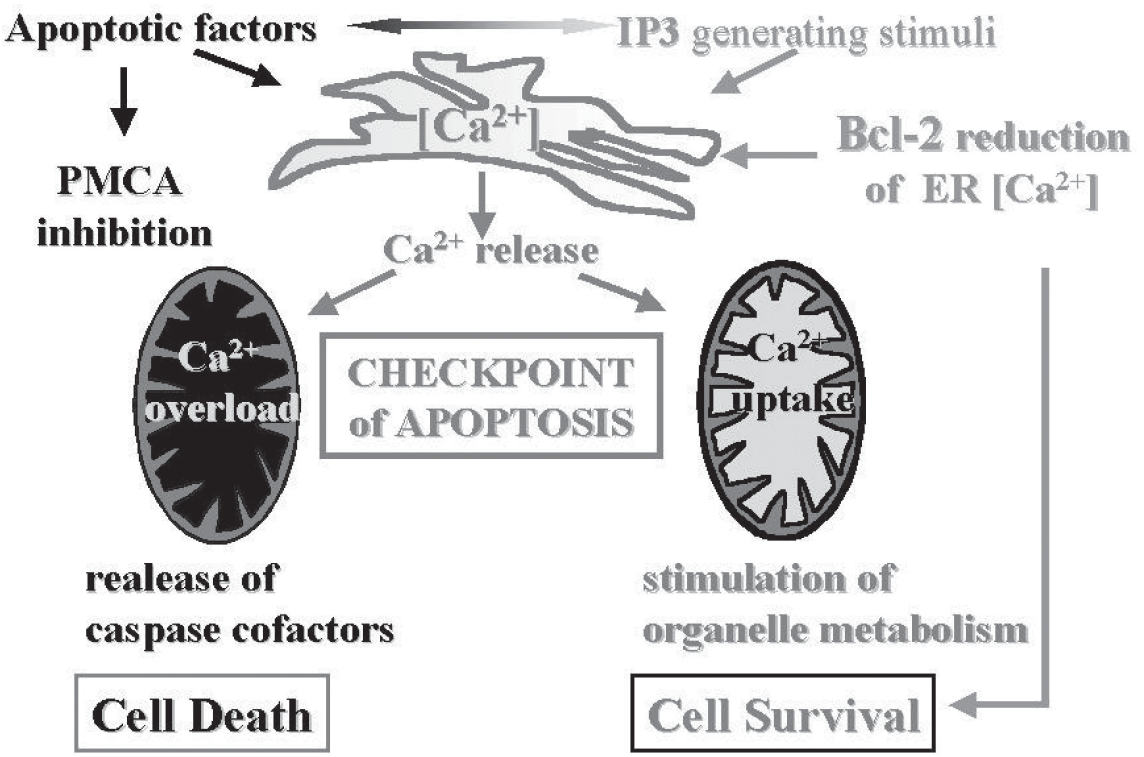

Figure 3. Mitochondria: checkpoint of apoptosis. The figure shows the role of mitochondrial calcium handling in determining cell death or survival. Apoptotic factors and physiological stimuli both act on calcium signaling, but exert opposite effects. The antiapoptotic protein Bcl-2 promotes cell survival by reducing ER Ca^{2+} level and consequently mitochondrial Ca^{2+} overload. Mitochondrial Ca^{2+} accumulation thus acts as an intracellular checkpoint to switch from physiological metabolic regulation to induction of apoptosis.

CONCLUSIONS

Much remains to be known about mitochondrial Ca^{2+} homeostasis, starting from the complete lack of molecular definition (neither the channels, nor the modulators, nor the scaffolding proteins of the ER/mitochondria contacts have been identified). The participation of mitochondria in key Ca^{2+} mediated events of the cell's life and death makes the forthcoming experimental effort potentially highly rewarding.

ACKNOWLEDGMENTS

This work was supported by grants from Telethon- Italy (Grants no. 1285 and GTF02013), the Italian Association for Cancer Research (AIRC), the Human Frontier Science Program, the Italian University Ministry (MURST and FIRB) and the Italian Space Agency (ASI) to R.R.

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