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Proapoptotic RECS1: a requisite gateway to lysosomal dysfunction and death

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For a long time since their discovery by Christian de Duve in the 1950s, lysosomes have been referred to almost exclusively as passive garbage bags; the endpoint in the degradation of intra- and extracellular cargo. The catabolic function of lysosomes is accomplished by an array of more than 60 acid hydrolases, which together break down a wide variety of biological macromolecules, including proteins, lipids, carbohydrates, and nucleic acids, for reutilization in the metabolic processes of the cell. For their optimal function, these enzymes require an acidic intraluminal pH of ~4.5, which is maintained by the joint action of a proton pump, the vacuolar H^+-ATPase, and several ion channels embedded in the lysosomal limiting membrane. Nowadays, lysosomes are envisioned as complex signaling hubs, integrating diverse stimuli about the cell's metabolic status to coordinate different adaptive responses (Ballabio and Bonifacino, 2020). The lysosome can also induce cell death signals in response to certain conditions, such as infections and treatment with lysosomotropic drugs, which leads to lysosomal membrane permeabilization (LMP) and the release of cathepsins, resulting in lysosomal-mediated cell death (**Figure 1A**, left). Lysosomes are also important intracellular calcium reservoirs. Lysosomal calcium plays essential functions in several cellular processes, such as lysosomal fusion with other vesicles, lysosomal biogenesis, and exocytosis (**Figure 1A**, right). In addition, lysosomal calcium is critical for lysosomal acidification, probably through the establishment of physical contacts with the endoplasmic reticulum. As a signaling molecule, calcium release from the lysosome through the transient receptor potential cation channel, mucolipin subfamily member 1 (TRPML1) activates the autophagic signaling pathway through the transcription factor EB (TFEB), which upregulates genes involved in autophagy and lysosomal biogenesis. Only three main types of lysosomal Ca^{2+} channels have been identified: the transient receptor potential channels of the mucolipin family, two-pore channels (TPC), and the trimeric Ca^{2+} two-transmembrane channel $P2X_4$. However, the lysosomal membrane comprises dozens of integral and peripheral proteins of unknown functions. The identification of new regulators of lysosomal biology is essential to better understand the role of lysosomes in the global regulation of adaptive and pro-dead responses, and their close connection to cell metabolism.

RECS1 (responsive to centrifugal force and shear stress 1) [also known as transmembrane bcl-2-associated X protein (BAX) inhibitor motif containing 1 (TMBIM1)] is a member of the TMBIM superfamily, which consists of at least six evolutionarily conserved proteins with homologs in viruses, bacteria and plants. The proteins of the TMBIM family localize to different intracellular membranes, including the endoplasmic reticulum, Golgi apparatus, and mitochondria, and they regulate stress-induced cell death and calcium homeostasis (Rojas-Rivera and Hetz, 2015). The resolution of the crystal structure of the TMBIM ortholog from *Bacillus subtilis*, BsYetJ, together with additional functional studies in human cells, have revealed that the proteins of the TMBIM family are seven-transmembrane, pH-sensitive calcium channels (Chang et al., 2014). RECS1 localizes to endosomes and lysosomes, suggesting that it may be involved in the regulation of lysosomal calcium homeostasis. RECS1 also regulates apoptosis in response to external stimuli; raising the possibility that its location at the lysosome membrane is implicated in the regulation of cell death. In our recent study (Pihan et al., 2021a), we have uncovered a novel function for RECS1 in the regulation of lysosomal pH, calcium homeostasis, and cell death. RECS1 overexpression triggered cell death through a crosstalk with the canonical mitochondrial pathway of apoptosis. These results suggested the identification of the first proapoptotic component of the TMBIM family.

One of the main problems to accurately measure lysosomal intraluminal calcium concentration is that the affinity of commonly used calcium fluorescent probes (i.e. Fura-2) varies non-linearly with proton concentration at pH below 6. To circumvent this issue, calcium and pH must be determined simultaneously in each lysosome, allowing the precise assessment of the effects of luminal pH on the affinity of the calcium probe (K_d) (Christensen et al., 2002). Using a simplified version of this method (Pihan et al., 2021b), we measured lysosomal pH and calcium in cells overexpressing RECS1. We found that RECS1 overexpression increased lysosomal acidification, correlating with increased lysosomal intraluminal calcium concentration. These results suggest that RECS1 may be a novel lysosomal calcium channel, regulating resting lysosomal Ca^{2+} and H^+ concentrations (**Figure 1B**, left). To directly determine RECS1's channel conductance, we expressed the protein in *Xenopus laevis* oocytes and carried out electrophysiological studies at different pH values. Interestingly, we found that RESC1 exhibited spontaneous single-channel current spikes at neutral pH, which decreased dramatically when the pH was lowered to 6.5. Our results showed that RECS1 is permeable to Ca^{2+} and Na^+, identifying a new lysosomal cation channel that regulates intraluminal pH and calcium concentration. Interestingly, as also shown in other members of the TMBIM family, the calcium conductivity of RECS1 is regulated by a conserved di-aspartyl pH sensor motif contained in its C-terminal domain. Ablation of this motif by mutagenesis significantly reduces the conductivity of the RECS1 channel. What cellular processes are regulated by RECS1 through calcium signals? We found that RECS1 overexpressing cells showed an increased accumulation of autophagosomes and a higher susceptibility to stress.

Lysosomal-mediated cell death pathways are poorly understood. However, lysosomes are considered an attractive therapeutic target for cancer, because the induction of LMP represents an effective strategy against a variety of different cancers. Since RECS1 is a member of the TMBIM family of apoptosis regulators and localizes to the lysosome, we assessed its function in the regulation of cell death triggered by different stress conditions. To this end, we conducted an

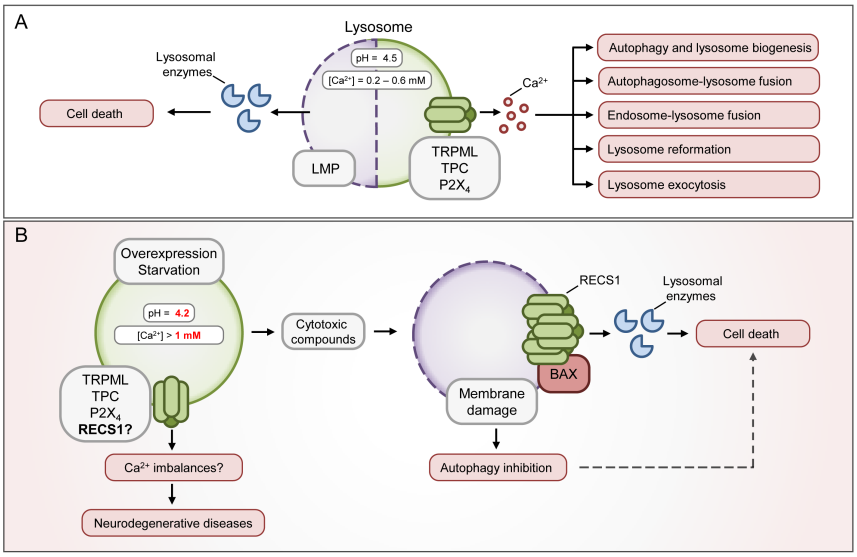

Figure 1 | **The newly discovered functions of RECS1 in the regulation of lysosomal calcium and cell death.** (A) Lysosomes are membrane-enclosed cytosolic organelles, functioning as the main intracellular catabolic compartment. In the lysosomal lumen, biomolecules are degraded by hydrolase enzymes that function at very acidic pH (~4.5). Under stress conditions, LMP leads to the cytosolic translocation of lysosomal enzymes causing cell death. Lysosomes are also intracellular calcium reservoirs. Lysosomal calcium signaling is mediated by at least three different families of calcium channels and is essential for many processes, including lysosomal fusion with different organelles and the induction of the autophagic pathway. (B) RECS1 overexpression or starvation results in decreased lysosomal pH and increased intraluminal calcium concentration, leading to imbalances in lysosomal calcium homeostasis. Abnormal calcium signaling is involved in the etiology of many diseases, including lysosomal storage disorders, neurodegenerative diseases and cancer. RECS1 overexpression sensitizes cells to various chemotherapeutic drugs, including the lysosomotropic agents CQ and HCQ. The treatment of RECS1 overexpressing cells with these drugs correlates with BAX translocation to the lysosomal membrane and LMP, leading to lysosomal-dependent cell death. Lysosomal membrane damage also results in autophagy inhibition, which under certain conditions, can lead to cell death. BAX: Bcl-2-associated X protein; CQ: chloroquine; HCQ: hydroxychloroquine; LMP: lysosomal membrane permeabilization; P2X4: trimeric Ca^{2+} two-transmembrane channel; RECS1: responsive to centrifugal force and shear stress 1; TPC: two-pore channel; TRPML1: transient receptor potential channels of the mucolipin family 1.

unbiased screening with a library of commonly used chemotherapeutic compounds. We found that RECS1 overexpression sensitized cells to a variety of different drugs, including microtubule destabilizers, while inhibiting the effects of DNA damage-inducing agents. However, the most potent sensitizing compounds were chloroquine (CQ) and hydroxychloroquine (HCQ), two lysosomotropic agents that accumulate in the lysosome and lead to increased lysosomal swelling, membrane rupture, and cell death (**Figure 1B**, right). Moreover, we showed that only cells overexpressing RECS1 were sensitized to these drugs, while wild-type cells were completely resistant. To gain mechanistic insights into RECS1's function, we investigated whether CQ treatment triggers LMP in RECS1 overexpression cell lines. We found that RECS1 induced LMP in response to CQ treatment. Of note, we observed the translocation of the proapoptotic protein BAX to the lysosomal membrane. BAX is a member of the BCL-2 family of apoptotic regulators that forms pores in the mitochondrial outer membrane during the intrinsic pathway of apoptosis, and may also trigger LMP (Bové et al., 2014). These results indicated that RECS1 triggers lysosomal-mediated death in cells treated with lysosomotropic agents and chemotherapeutical compounds. Interestingly, these results suggest that the expression levels of RECS1 –and possibly other lysosomal calcium channels –may determine the sensitivity thresholds of cancer cells to lysosomotropic agents, a hypothesis we are currently exploring. Currently, CQ and HCQ are the only clinically available drugs used to inhibit autophagy and are being used as co-adjuvant in combination with a wide variety of chemotherapeutic compounds for various types of cancers such as glioblastoma, melanoma, and pancreatic adenocarcinoma (Levy et al., 2017). Many different cancers rely on the induction of autophagy to adapt and scavenge for nutrients under conditions where inadequate tumor vascularization can result in limited nutrient availability. For example, the upregulation of the TFEB allows cancer cells to keep the necessary supply of amino acids for growth even in unfavorable conditions (Perera et al., 2015).

Imbalances in lysosomal acidification and calcium homeostasis are implicated in the etiology of various types of neurological and autoimmune diseases. For example, aberrant lysosomal calcium signaling has been implicated as a pathological driver for lysosomal storage disorders; a heterogeneous group of pathologies in which, due to mutations in different lysosomal enzymes, specific substrates accumulate in the lysosomal lumen. Newman Pick, type C1 (NPC1) is a rare lysosomal storage disorder caused by mutations in NPC1, a lysosomal transmembrane protein that shuttles cholesterol to the cytosol, resulting in its abnormal accumulation inside lysosomes and decreased luminal calcium (Lloyd-Evans et al., 2008). Interestingly, the chelation of lysosomal luminal calcium in healthy cells resulted in a complete phenocopy of NPC1 disease, suggesting that defects in lysosomal calcium may directly contribute to its pathogenesis (Lloyd-Evans et al., 2008). Mutations in *CLN3*, a six transmembrane lysosomal protein of unknown function, causes Juvenile neuronal ceroid lipofuscinosis (CLN3 disease), a genetic neurometabolic disorder characterized by the development of seizures, vision loss, and progressive neurodegeneration. One of the salient intracellular features of this disease is the higher resting concentration of lysosomal calcium, which correlates with altered autophagy due to impaired membrane fusion (Lloyd-Evans and Waller-Evans, 2020). CLN3 disease is the only known lysosomal disease associated with increased lysosomal calcium. We found that overexpression of RECS1 resulted in increased resting lysosomal calcium concentration along with altered autophagy. Lysosomal calcium aberrations have also been reported in several neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and frontotemporal dementia. How do calcium imbalances in the lysosome lead to neuronal cell death and pathology? It is known that, in familial forms of Alzheimer´s disease, mutations in the gene that codes for presenilin 1 (PS1) –the catalytic subunit of the γ-secretase complex, which cleaves the amyloid precursor protein, yielding amyloid β peptides –leads to impairments in autophagy, causing the accumulation of incompletely degraded substrates in the lysosome. In fact, PS1 is believed to promote the maturation and lysosomal localization of the vacuolar H^+-ATPase V0a1 subunit, which is essential for proper lysosomal acidification. However, mutations in PS1 lead to V0a1 instability and degradation, resulting in increased lysosomal pH and lysosomal calcium depletion through the hyperactivation of the H^+-sensitive lysosomal calcium channel TPRLM1 (Lee et al., 2015). Low levels of lysosomal calcium impair the fusion of autophagosomes with lysosomes while high lysosomal pH inhibits the activity of lysosomal catabolic enzymes, which leads to an overall impaired breakdown of autophagy substrates and the consequent occurrence of pathological features (Coen et al., 2012). Thus, lysosomal calcium and pH are direct drivers of pathological features in several neurological diseases, probably through the modulation of the autophagic pathway. The accumulation of abnormal lysosomes and their permeabilization has also been reported in Parkinson´s disease (Bové et al., 2014). Thus, restoring aberrant lysosomal calcium signaling or blocking LMP with pharmacological approaches has the potential to reverse several pathophysiological features of neurodegenerative diseases. The design and identification of RECS1 channel inhibitors represent a new putative therapeutic target as previously reported for BAX (Hetz et al., 2005).

In conclusion, aberrations in lysosomal calcium signaling are implicated in the etiology of many diseases, including neurological diseases and cancer. The discovery of new lysosomal calcium channels opens up the possibility to uncover previously unanticipated functions for these organelles in cell physiology, and to deepen our understanding of their connection to important cellular processes such as autophagy and cell death.

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