# The journey of Ca2+ through the cell - pulsing through the network of ER membrane contact sites

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REVIEW

# The journey of $Ca^{2+}$ through the cell – pulsing through the network of ER membrane contact sites

**Tom Cremer, Jacques Neefjes and Ilana Berlin***


## ABSTRACT

Calcium is the third most abundant metal on earth, and the fundaments of its homeostasis date back to pre-eukaryotic life forms. In higher organisms, $Ca^{2+}$ serves as a cofactor for a wide array of (enzymatic) interactions in diverse cellular contexts and constitutes the most important signaling entity in excitable cells. To enable responsive behavior, cytosolic $Ca^{2+}$ concentrations are kept low through sequestration into organellar stores, particularly the endoplasmic reticulum (ER), but also mitochondria and lysosomes. Specific triggers are then used to instigate a local release of $Ca^{2+}$ on demand. Here, communication between organelles comes into play, which is accomplished through intimate yet dynamic contacts, termed membrane contact sites (MCSs). The field of MCS biology in relation to cellular $Ca^{2+}$ homeostasis has exploded in recent years. Taking advantage of this new wealth of knowledge, in this Review, we invite the reader on a journey of $Ca^{2+}$ flux through the ER and its associated MCSs. New mechanistic insights and technological advances inform the narrative on $Ca^{2+}$ acquisition and mobilization at these sites of communication between organelles, and guide the discussion of their consequences for cellular physiology.


**KEY WORDS: Calcium, ER, Membrane contact sites, Mitochondria, Endosome**

## Introduction

Owing to its distinct chemical properties, $Ca^{2+}$ crucially contributes to cellular homeostasis, and responsiveness to its fluctuations forms the basis of excitability in a variety of cell types (Carafoli and Krebs, 2016; Kuo and Ehrlich, 2015). To maintain a dynamic range of influence over essential biological processes, intracellular $Ca^{2+}$ is sequestered into organellar stores and released on demand, establishing bursts of local concentration gradients (Filadi and Pozzan, 2015). By far the largest $Ca^{2+}$ store in most cell types is the endoplasmic reticulum (ER). Extending from the nuclear envelope towards the outer edges of the cell, the ER forms a dynamic and agile membranous network (Grigoriev et al., 2008; Guo et al., 2018; Rodriguez-Garcia et al., 2020; Zhang et al., 2010). Sheet-like rough ER (Nixon-Abell et al., 2016; Schroeder et al., 2019), home to ribosomes and protein translocation machinery, concentrates in the perinuclear region of the cell, while the tubular smooth ER, which features functionally diverse nanodomains, reaches far into the cell periphery and possesses a high degree of curvature (Gao et al., 2019; Goyal and Blackstone, 2013; Voeltz et al., 2006; Wang et al., 2016b), well-suited for interactions with other organelles (Phillips and Voeltz, 2016; Westrate et al., 2015). These interactions are established and regulated through specialized membrane contact sites (MCSs) and localize to phase-separated domains within opposing organellar membranes (King et al., 2020), where they serve as terminals for influence and exchange (Prinz et al., 2020; Wu et al., 2018). In this Review, we discuss how the interactive ER network funnels $Ca^{2+}$ flow from the extracellular space towards other organelles (Fig. 1). Focusing on pathways commonly used by a variety of cell types, we provide perspectives on the $Ca^{2+}$-dependent processes that are engaged at ER MCSs, including mitochondrial energy production, initiation of autophagy, and trafficking of endocytic cargoes.

## The rite of passage – $Ca^{2+}$ entry at ER–PM MCSs

Uptake of $Ca^{2+}$ from extracellular space is managed by store-operated $Ca^{2+}$ entry (SOCE) at MCSs formed between the ER and plasma membrane (PM) (Fig. 2). SOCE is induced when the membrane-resident R-like ER kinase (PERK; also known as EIF2AK3) detects a rise in cytosolic $Ca^{2+}$ (van Vliet et al., 2017). Activation of PERK initiates cortical actin rearrangements and facilitates the ER approaching the PM at sites designated by the tether Gram-domain containing protein 2A (GRAMD2a) (Besprozvannaya et al., 2018; van Vliet et al., 2017). At the same time, ER transmembrane stromal interaction molecule 1 (STIM1) senses a drop in ER $Ca^{2+}$ levels and responds by oligomerizing at the ER–PM interface to bind and activate the PM channel $Ca^{2+}$ release-activated $Ca^{2+}$ modulator 1 (Orai1) (Gudlur et al., 2018; Liou et al., 2005; Prakriya and Lewis, 2015; Roos et al., 2005). Subsequently, extracellular $Ca^{2+}$ can enter the ER through the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump (Manjarrés et al., 2010). Assembly of these molecular players at ER–PM MCSs not only averts global increases in cytosolic $Ca^{2+}$, but may also facilitate lipid exchange through cooperative action of extended synaptotagmins (ESYTs) at these locations (Saheki et al., 2016; Yu et al., 2016). ESYT proteins anchor in the ER membrane and harbor multiple C2 domains that bind the phosphoinositide phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], which is enriched at the PM (van Meer and de Kroon, 2011). The same domains can also bind $Ca^{2+}$ (as shown in the case of ESYT1) and thus could be involved in SOCE (Idevall-Hagren et al., 2015; Yu et al., 2016). Indeed, ESYT1 has recently been shown to organize and stabilize STIM1 into ring-like structures at ER–PM MCSs, thereby potentiating SOCE (Kang et al., 2019). In addition, ESYTs contain a synaptotagmin-like mitochondrial-lipid-binding (SMP) domain; this is thought to mediate the exchange of PM-derived diacylglycerol (DAG) for PI(4,5)P2 coming from the ER (Saheki et al., 2016; Saheki and De Camilli, 2017). Consequently, ESYTs can reset the lipid composition of the PM in response to phospholipase C (PLC)-mediated hydrolysis of PI(4,5)P2 into PI(4)P and the second messenger inositol trisphosphate (IP3), which for instance occurs upon activation of G-protein-coupled receptors by

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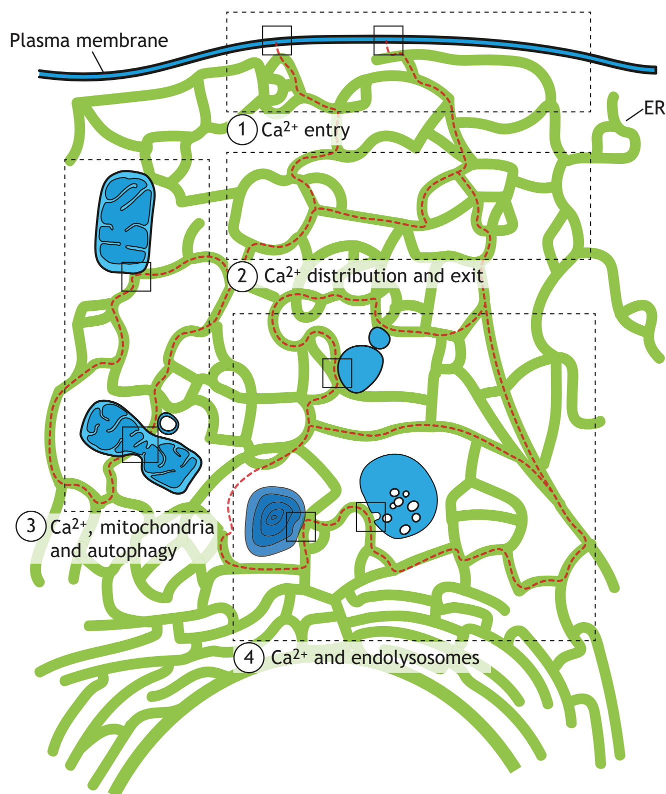


**Fig. 1. The journey of $Ca^{2+}$ through the ER and its membrane contact sites.** Overview of intracellular $Ca^{2+}$ movement and its consequences for organellar physiology with a focus on $Ca^{2+}$ dynamics at ER membrane contact sites (MCSs; indicated with small squares). (1) Entry of $Ca^{2+}$ into the ER through ER-PM MCSs; (2) $Ca^{2+}$ activities within the ER lumen while being transported towards exit sites at MCSs; (3) $Ca^{2+}$ exchange at MCSs – the ER engages mitochondria to regulate cellular bioenergetics, mitochondrial remodeling and autophagy initiation; and (4) $Ca^{2+}$-dependent processes such as fusion, refilling and intraluminal vesicle fomation at MCSs between the ER and the endolysosomal system.

$Ca^{2+}$ or other ligands (Leybaert and Sanderson, 2012; Saheki et al., 2016; Saheki and De Camilli, 2017). Although ESYT-dependent contacts are not required for SOCE (Giordano et al., 2013), local $Ca^{2+}$ influx at ER–PM contact sites increases ESYT function (Idevall-Hagren et al., 2015) and thus creates an elegant feedback mechanism to restore cellular homeostasis following IP3-driven $Ca^{2+}$ depletion from the ER.

## $Ca^{2+}$ homeostasis on the home front

While a well-functioning $Ca^{2+}$ store can acquire sufficient $Ca^{2+}$ to accommodate global cellular demand, swift transport is needed to deliver $Ca^{2+}$ to its ER exit channels, as demonstrated in polarized cells and neurons, as well as non-excitable cells (Petersen et al., 2017). For example, in secretory pancreatic acinar cells, $Ca^{2+}$ has been shown to travel over long distances in mere seconds (Park et al., 2000); because ATP depletion hampers trafficking of ER lumen constituents, such as chaperone-client protein complexes (Barrow et al., 2008; Nehls et al., 2000), it stands to reason that flow of $Ca^{2+}$ through the ER likely relies on an active process. Indeed, using super-resolution imaging of photoconvertible fluorescent proteins in various cell types, it was recently shown that localized, asynchronous and ATP-dependent ER-tubule contraction events drive luminal flow (Holcman et al., 2018). Interestingly, inhibition of the SERCA-mediated $Ca^{2+}$ influx promotes these contractions (Holcman et al., 2018), prompting the question of whether $Ca^{2+}$ simply ‘surfs’ the ER network, or rather controls the waves. In this context, the luminal spacer cytoskeleton-linking membrane protein 63 (CLIMP63; also known as CKAP4) was found to interact with the $Ca^{2+}$-binding protein calumenin and to cooperatively regulate the width of the luminal ER (Shen et al., 2019) (Fig. 2). Calumenin attains its functionally folded state through low-affinity $Ca^{2+}$ binding via multiple EF-hand domains (Mazzorana et al., 2016; Yabe et al., 1997). Effectively, calumenin is a high capacity $Ca^{2+}$ sensor; in response to small changes in local $Ca^{2+}$, it might regulate the interactions between CLIMP63 and microtubules (Shen et al., 2019) that are needed to facilitate ER tubule contractions through an as-yet-unclear mechanism. An appealing model would involve the generation of flow through contraction of sections of ER tubules, depending on the local intraluminal $Ca^{2+}$ concentration.

In addition to tubule contraction, $Ca^{2+}$ ‘wiring’ mediated by polymeric forms of the $Ca^{2+}$-buffering protein calsequestrin (which has CASQ1 and CASQ2 forms) may offer an alternative mechanism for fast $Ca^{2+}$ mobility throughout the sarcoplasmic reticulum (SR) of muscle cells by providing an intraluminal framework for the diffusion of $Ca^{2+}$ in one dimension (MacLennan and Reithmeier, 1998; Park et al., 2003; Royer and Rios, 2009; Zhu et al., 2012) (Fig. 2). Additionally, calsequestrin, interacts directly with the ryanodine receptor (RyR) $Ca^{2+}$ channel in muscle cell triads (Handhle et al., 2016). While calsequestrin is found predominantly in muscle tissue, it is also expressed in many other tissue types (Wang et al., 2015; Zhu et al., 2012) and could therefore similarly influence $Ca^{2+}$ trafficking in non-specialized cells. Interestingly, calsequestrin can interact with and stabilize monomeric STIM1 (Wang et al., 2015), which binds to the microtubule plus-end tracking protein end-binding 1 (EB1; also known as MAPRE1) that helps extend the ER membrane along growing microtubules (Grigoriev et al., 2008; Rodriguez-Garcia et al., 2020). This suggests that polymeric calsequestrin ‘wires’ could reach into newly formed EB1-positive ER tubules. Depletion of luminal ER $Ca^{2+}$ negatively regulates ER tubule extension by partially uncoupling STIM1 foci from growing EB1-positive microtubule plus-ends to initiate SOCE (Chang et al., 2018; Grigoriev et al., 2008). In this way, $Ca^{2+}$ release through ER exit sites may limit excessive ER network protrusion into $Ca^{2+}$-demanding areas of the cell, hence averting hyperdepletion of ER $Ca^{2+}$ stores (Grigoriev et al., 2008). At the same time, a proportion of STIM1 remains sequestered at EB1 foci during ER $Ca^{2+}$ release and thus is unavailable to facilitate new $Ca^{2+}$ entry into the ER via SOCE (Chang et al., 2018). This and other feedback mechanisms aimed at avoiding $Ca^{2+}$ ‘overfilling’ of the ER lumen (Konno et al., 2012; Philippe et al., 2017; Wang et al., 2015) highlight the importance of balancing $Ca^{2+}$ availability with the dangers of oversupply, which have been linked to apoptotic cell death and neurological disorders (Sammels et al., 2010b).

As $Ca^{2+}$ travels through the extensive labyrinth of the ER lumen, it is harnessed to manage a variety of luminal ER activities (Fig. 2). Of all its functions, the ER is best known for the biogenesis of secretory and membrane proteins, which is mediated by a wide array of luminal chaperones (Ellgaard et al., 2016). The majority of these chaperones, including calnexin, calreticulin and grp78 (also known as BiP and HSPA5) depend on $Ca^{2+}$, and the folding of $Ca^{2+}$-binding proteins, such as integrins, is directly influenced by $Ca^{2+}$ levels in the ER (Philippe et al., 2017; Tiwari et al., 2011). Moreover, correct (re)folding of disulfide-bond-containing proteins relies on a fine-tuned glutathione redox balance that is governed by the exchange of reduced glutathione (GSH) and $Ca^{2+}$ with the

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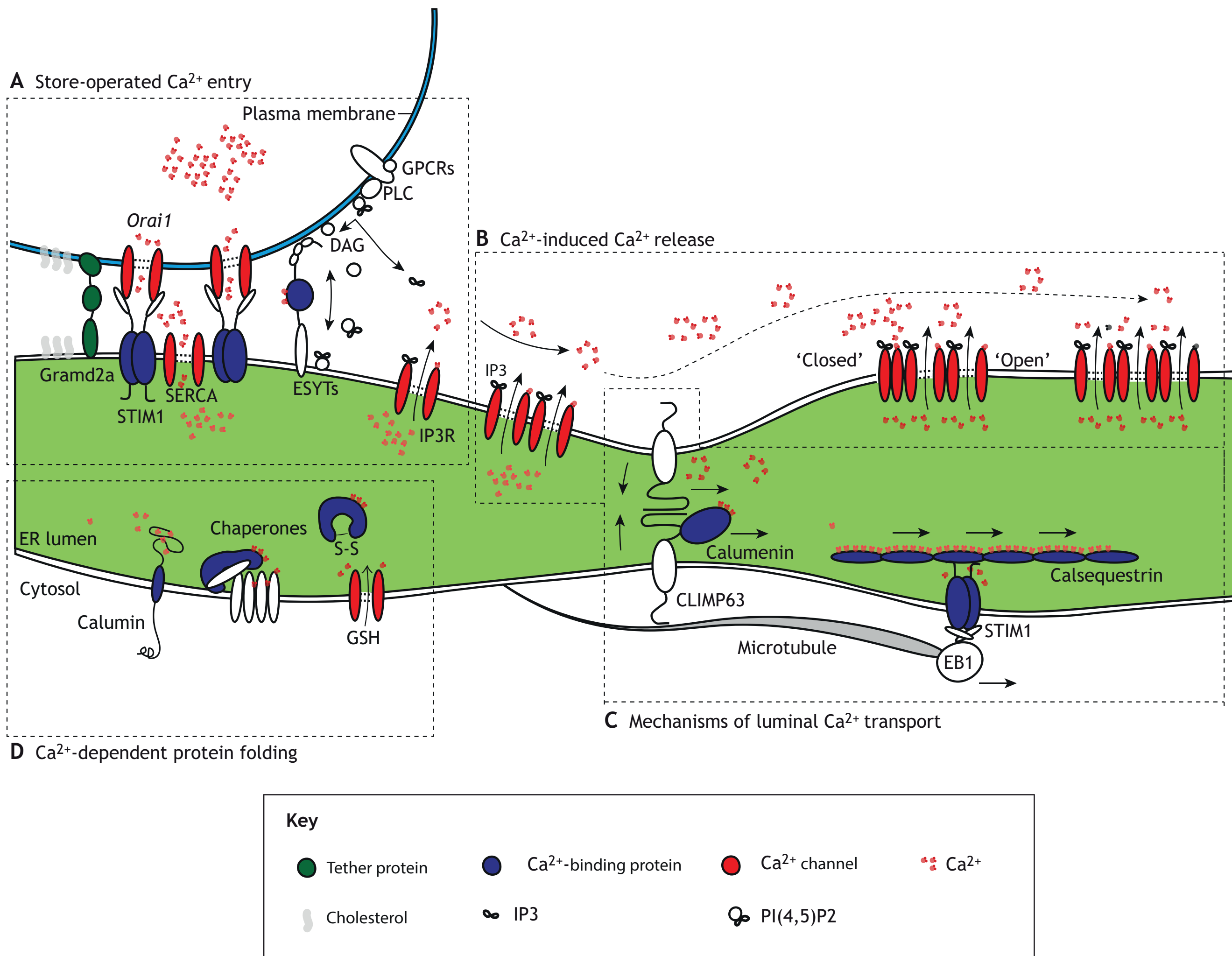


**Fig. 2. Store-operated $Ca^{2+}$ entry at ER-PM MCSs and $Ca^{2+}$ activities in the ER.** (A) Store-operated $Ca^{2+}$ entry. IP3 signaling induces $Ca^{2+}$ release from ER-embedded IP3Rs. The rise in cytoplasmic $Ca^{2+}$ in turn triggers activation of store-operated $Ca^{2+}$ entry (SOCE) at ER-PM MCSs, designated by GRAMD2a. SOCE depends on the activity of the Orai1 and SERCA $Ca^{2+}$ channels, which are coordinated by the $Ca^{2+}$ sensor STIM1. Simultaneously, the $Ca^{2+}$ microenvironment at ER-PM MCS enhances ESYT-mediated lipid exchange to reconstitute PM lipid composition after G-protein coupled receptor activation and PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] into diacylglycerol (DAG) and IP3. (B) $Ca^{2+}$-induced $Ca^{2+}$ release. IP3-primed IP3R clusters elicit $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) to propagate $Ca^{2+}$ signals further into the cell. IP3Rs open at moderate $Ca^{2+}$ availability and close upon enhanced $Ca^{2+}$ accumulation to generate $Ca^{2+}$ 'puffs'. (C) Mechanisms of luminal $Ca^{2+}$ transport. To meet changing cellular demands, the ER network is remodeled through STIM1-mediated interactions with EB1 on growing microtubules. $Ca^{2+}$ flow throughout the ER is governed by ER tubule contractions that may involve $Ca^{2+}$ binding by calumenin and CLIMP63. Polymeric calsequestrin fibers enhance the $Ca^{2+}$ diffusion rate in the SR of muscle cells and potentially in other cell types. Polymeric forms of calsequestrin may interact with inactive STIM1 to extend into newly forming ER tubules. (D) $Ca^{2+}$-dependent protein folding. ER-resident processes, including chaperone-assisted protein folding and co-translational insertion of transmembrane helices, depend on $Ca^{2+}$ availability, either involving direct $Ca^{2+}$ binding or association with a $Ca^{2+}$-binding factor, such as calumin.

cytoplasm (Lizak et al., 2020). When extensive efforts of chaperones are insufficient for a protein to attain its correctly folded state, it can be targeted for degradation by the proteasome through ER-associated degradation (ERAD) (Fregno and Molinari, 2019). Here, $Ca^{2+}$-binding calumin (also known as CCDC47) interacts with several members of the ERAD machinery (Yamamoto et al., 2014), and, recently, calumin and transmembrane and coiled-coil domains 1 (TMCO1) were identified as essential members of the PAT complex, which aids in co-translational insertion of a wide variety of integral membrane proteins into the ER membrane (Chitwood and Hegde, 2020; McGilvray et al., 2020). These findings point to a major role for $Ca^{2+}$ in protein folding and degradation from the ER. However, enabling these ER-inherent processes is only the beginning, as $Ca^{2+}$ exits the ER to stimulate dynamic series of molecular events elsewhere. Interestingly, $Ca^{2+}$ exit sites often coincide with contacts the ER makes with other organelles, such as mitochondria and endosomes. Recent insights into the mechanisms of $Ca^{2+}$ release at these MCSs and the consequences thereof are discussed in the next section.

## MCSs – an exit strategy

$Ca^{2+}$ exits the ER mainly through two types of release channels, the aforementioned RyRs or inositol-1,4,5-phosphate receptors (IP3Rs) (Prole and Taylor, 2019; Santulli et al., 2017); however, new $Ca^{2+}$ exit channels continue to be discovered (Wang et al., 2016a). RyRs and IP3Rs exhibit ~40% sequence identity and have likely evolved from a common ancestor (Alzayady et al., 2015), yet, they display

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distinct physiological characteristics (Santulli et al., 2017). Specifically, RyRs mainly evoke increases in $Ca^{2+}$ levels from the SR of muscle tissue, which are required for excitation–contraction coupling (Kuo and Ehrlich, 2015; Santulli et al., 2017). Since these processes are highly specialized, and several excellent reviews have been written on this topic, we will not delve into RyR biology here (Eisner, 2014; Santulli et al., 2018). On the other hand, IP3Rs function in a variety of tissues, although individual family members differ in cell type expression, affinity for IP3 and capacity to sustain $Ca^{2+}$ oscillations (Prole and Taylor, 2019). Additionally, other ER $Ca^{2+}$ exit channels might work in conjunction with IP3Rs and RyRs to amplify intracellular $Ca^{2+}$ signals, as has been shown for renal cell-specific polycystin-2 in the context of autophagy induction (Anyatonwu et al., 2007; Peña-Oyarzun et al., 2020; Sammels et al., 2010a). As IP3Rs have been reported at contacts between the ER and other organelles, their organization and activation are discussed in detail here.

IP3Rs form large tetrameric structures composed of 300 kDa subunits embedded in the ER membrane, with each subunit featuring six transmembrane regions. These receptors respond to the archetypical second messenger IP3 (Leybaert and Sanderson, 2012), which has been suggested to prime, and thus cluster, IP3Rs at a considerable distance from the PM (Dickinson et al., 2016; Taufiq Ur et al., 2009). IP3Rs are co-regulated by cytosolic $Ca^{2+}$ through a principle known as $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (Fig. 2) (Prole and Taylor, 2019), whereby IP3Rs remain closed at low cytosolic $Ca^{2+}$ levels, and are activated in response to increasing $Ca^{2+}$ to allow ER $Ca^{2+}$ egress. However, once local $Ca^{2+}$ accumulation surpasses a given threshold, IP3Rs become auto-inhibited. The resulting waves of activation and inhibition generate short $Ca^{2+}$ 'puffs', which may, through diffusion, activate nearby clusters of ligand-primed IP3Rs (Vais et al., 2010). A proportion of these clusters is immobilized at ER–PM MCSs; here, they initiate oscillatory $Ca^{2+}$ signals, while also being optimally positioned to stimulate the SOCE to immediately replenish the ER with fresh $Ca^{2+}$ (Sampieri et al., 2018; Thillaiappan et al., 2017). In response to their activation, a fraction of IP3R clusters is downregulated through ubiquitylation and consequent proteasomal degradation that protects cells from an excessive $Ca^{2+}$ mobilization (Wright and Wojcikiewicz, 2016).

It is worth emphasizing, that once released into the cytosol, $Ca^{2+}$ is quickly buffered by a wide array of $Ca^{2+}$-binding proteins, such as calmodulin, so that only 1% of cytosolic $Ca^{2+}$ remains free (Schwaller, 2010). In this regard, IP3Rs create a microenvironment for $Ca^{2+}$ and $Ca^{2+}$-binding proteins to act as local messengers (Prole and Taylor, 2019). In the next sections, we will discuss how such microenvironments at ER MCSs influence the behavior of their associated organelles, including mitochondria and endolysosomes.

### Tuning cellular metabolism at ER–mitochondria MCSs

As far back as the early 1990s, co-fractionation experiments had shown that mitochondria-associated membranes (MAMs) contain ER components involved in lipid and $Ca^{2+}$ signaling (Vance, 1990). It is now appreciated that 2–5% of the mitochondrial surface in the cell is engaged in contacts with the ER (Friedman and Nunnari, 2014; Phillips and Voeltz, 2016). Tethers, such as the mitochondrial protein tyrosine phosphatase (PTP) interacting protein 51 (PTPIP51; also known as RMDN3) and ER-located vesicle-associated membrane protein-associated protein B (VAPB) can mediate opposition of their respective organelles (Gomez-Suaga et al., 2017), and new tethers for these types of inter-organellar contacts continue to be uncovered (Wu et al., 2018). Tethering of ER to mitochondria promotes $Ca^{2+}$ exchange into the intermembrane space between the outer and inner mitochondrial membranes (OMM and IMM, respectively) through the interactions between IP3Rs and mitochondrial voltage-dependent-anion channels (VDACs) (Gomez-Suaga et al., 2017) (Fig. 3). From here, $Ca^{2+}$ is shuttled into the mitochondrial matrix, mainly via the mitochondrial $Ca^{2+}$ uniporter (MCU) (De Stefani et al., 2016). In the ER membrane, activation of IP3Rs is positively regulated by the sigma1 receptor (Sigma1R) (Wu and Bowen, 2008), which is responsive to choline, the product of phosphatidylcholine (PC) cleavage mediated by the mitochondria-specific phospholipase D family member (mitoPLD; also known as PLD6). In this way, the choline signals can locally activate IP3Rs for $Ca^{2+}$ release at the ER–mitochondria MCS (Brailoiu et al., 2019; Gao and Frohman, 2012; Vance, 1990). Recently, gain-of-function mutations in IP3R have been shown to extend *C. elegans* lifespan (Burkewitz et al., 2020), highlighting the impact of regulated mitochondrial $Ca^{2+}$ homeostasis on cellular and organismal wellbeing.

Once shuttled from the ER–mitochondria MCS into the mitochondrial matrix, $Ca^{2+}$ takes on cofactor functions for a wide range of enzymatic reactions involved in ATP production (De Stefani et al., 2011; Glancy and Balaban, 2012) (Fig. 3). In exchange, mitochondria replenish the cytoplasm and ER with ATP (Cardenas et al., 2010; Yong et al., 2019), which can then also be used for $Ca^{2+}$ re-influx via SERCA. This interplay responds to changes in cellular energy demand, such as the stress induced by protein misfolding in the ER (Bravo et al., 2011). Acute ER stress increases the number of perinuclear ER–mitochondria MCSs, boosting the $Ca^{2+}$ and ATP exchange (Bravo et al., 2011; Yong et al., 2019). By contrast, a global rise in ATP demand results in an expansion of ER–mitochondria contacts throughout the cell (Bravo-Sagua et al., 2016), pointing to a spatial coordination of ER–mitochondria MCS formation in accordance with the type of cellular demand. While mitochondria are adept at a needs-based energy supply, a balanced $Ca^{2+}$ influx into these organelles is desired to avert negative consequences of mitochondrial overstimulation. For instance, excessive $Ca^{2+}$ exchange at ER–mitochondria MCSs, due to the accumulation of reactive oxygen species (ROS) or certain chemotherapy treatments, can induce a pro-apoptotic mitochondrial signature (De Stefani et al., 2012; Doghman-Bouguerra et al., 2016; Verfaillie et al., 2012). Moreover, upregulated MCU transporter levels in colorectal cancer (CRC) cells have been associated with poor prognosis of CRC patients (Liu et al., 2020). To maintain a healthy $Ca^{2+}$ homeostasis in mitochondria, $Ca^{2+}$ can be exported from the mitochondrial matrix by the mitochondrial $Na^+/Ca^{2+}$ exchanger (NCLX; also known as SLC8B1), generating intra-organellar oscillations that mimic those of cytoplasmic $Ca^{2+}$ (Samanta et al., 2018).

Mitochondrial form follows function (Favaro et al., 2019; Silva Ramos et al., 2016), and both are supported by dynamic processes of fusion and fission of these organelles, wherein $Ca^{2+}$ is a key participant (Fig. 3). Mitochondrial fusion and fission are controlled by ER-associated proteins and may occur sequentially to mix mitochondrial matrix constituents and repolarize metabolically inactive mitochondria (Abrisch et al., 2020). Although its mechanism remains incompletely resolved, mitochondrial fusion is mediated by either homo- or hetero-typic interactions between the mitofusin 1 and mitofusin 2 proteins on mitochondria and the ER, which are coupled to tethering of mitochondria to the ER membrane (Filadi et al., 2015; Legros et al., 2002; Mattie et al., 2018; Santel and Fuller, 2001). In contrast, mitochondrial fission involves coordination by the ER as well as the presence of late

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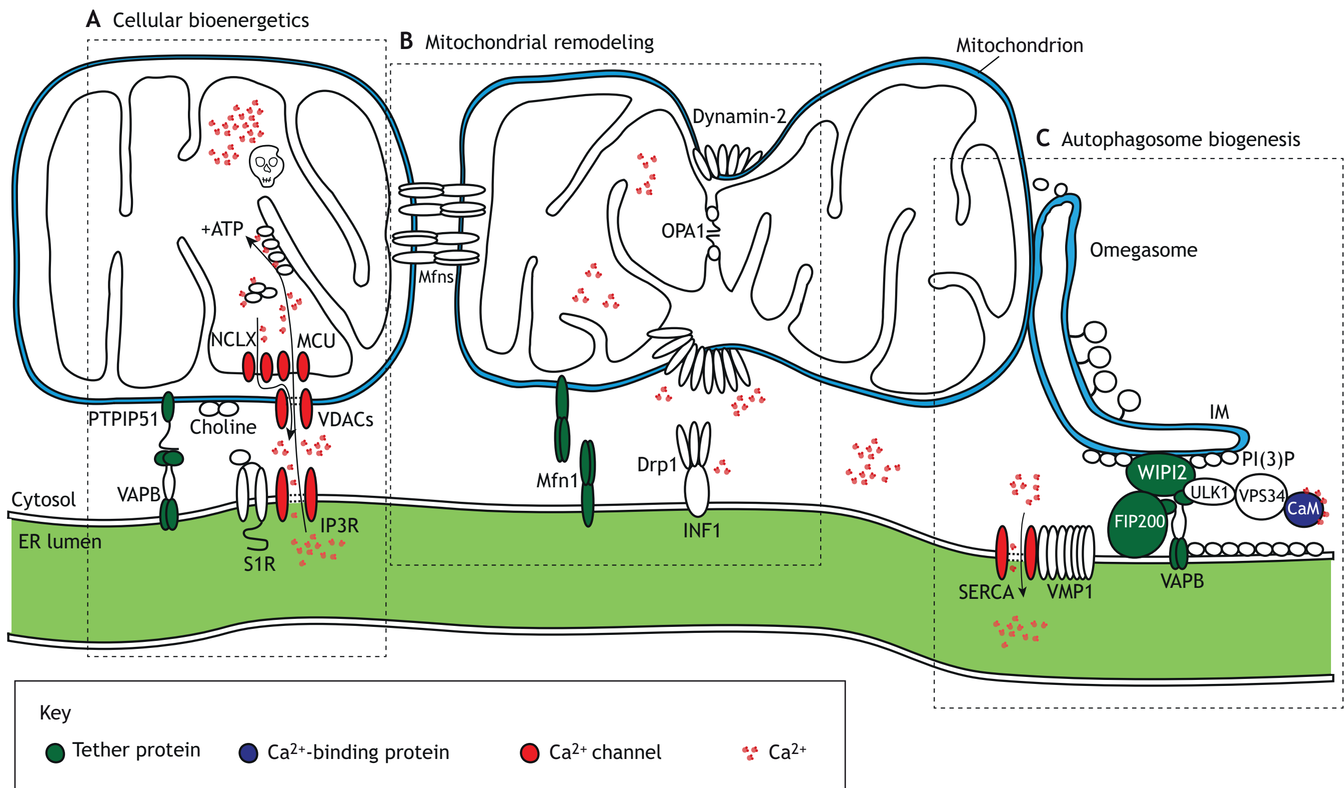


**Fig. 3. $Ca^{2+}$ exchange at ER-mitochondria MCSs regulates cellular bioenergetics, mitochondrial remodeling and autophagy biogenesis.** (A) Cellular bioenergetics. Membrane tethers formed by mitochondrial PTPIP51 and VAPB govern ER–mitochondrial coupling for $Ca^{2+}$ exchange through IP3R, VDACs and MCU channels in the ER, or the outer and inner mitochondrial membrane (OMM, IMM), respectively. Choline signaling may enhance IP3R activation through the sigma1 receptor. In the mitochondrial matrix, $Ca^{2+}$ activates multiple enzymes that stimulate ATP production. However, increased $Ca^{2+}$ exchange may result in a pro-apoptotic mitochondrial signature. Appropriate $Ca^{2+}$ levels are maintained by reflux out of the mitochondrial matrix by a $Na^{+}$-$Ca^{2+}$ exchanger (NCLX). (B) Mitochondrial remodeling. Repolarization of mitochondria depends on the fission and fusion machinery that localizes to ER–mitochondria MCSs. Fusion depends on homo-and hetero-typic interactions between mitofusins (Mfns), whereas fission relies on $Ca^{2+}$-mediated oligomerization of Drp1 on its receptor inverted furin 1 (INF1) and dynein-2, together with OPA1 present in the mitochondrial matrix. (C) Autophagosome biogenesis. Upon starvation, interactions of calmodulin (CaM) with the PI(3)P-producing kinase VPS34 drive ULK1-mediated autophagosome biogenesis, for which mitochondria may provide lipids. During its extension, the isolation membrane (IM) is dynamically tethered to the ER by VAPB, FIP200 and WIPI2. Phagophore closure is completed upon VMP1-SERCA-mediated $Ca^{2+}$ efflux from ER–IM MCSs.

endosomes/lysosomes and their GTPase Rab7a (Friedman et al., 2011; Wong et al., 2018). With regard to fission, the dynamin family member dynamin-related protein 1 (Drp1; also known as DNM1L) is thought to oligomerize on $Ca^{2+}$-activated inverted formin 2 (INF2) receptors located at the ER membrane prior to arriving at mitochondria (Ji et al., 2017, 2015), presumably via ER–mitochondria MCSs. Drp1 initializes constriction of OMM, whereupon fission is completed by the dynamin-2 GTPase (Lee et al., 2016). In contrast to Drp1, dynamin-2 activity appears to be inhibited by $Ca^{2+}$ (Cousin and Robinson, 2000), suggesting that $Ca^{2+}$ release from the ER at ER–mitochondria MCSs influences distinct stages of mitochondrial fission in different ways. In this context, IMM constriction follows INF2-induced $Ca^{2+}$ import and relies on the coordinated activation of another dynamin family member, optic atrophy 1 (OPA1) (Chakrabarti et al., 2017; Ge et al., 2020; Song et al., 2007). Here, at least three known mitochondrial proteases, responsive to different environmental stimuli, process OPA1 into fragments that cooperatively function in the final stages of IMM fusion (Ge et al., 2020). Collectively, these findings support a possible coupling between OMM fission and IMM constriction that is achieved through timely $Ca^{2+}$ signals.

By way of multiple interactions taking place at the ER–mitochondria MCS, $Ca^{2+}$ controls energy production and mitochondrial morphology, both in times of nutrient abundance and during periods of starvation (Fig. 3). Mitochondrial energy production is also intimately linked with the ability of the cell to digest its components through autophagy (Cardenas et al., 2010). For instance, sustained $Ca^{2+}$ import into mitochondria through IP3Rs inhibits autophagy, whereas loss of IP3Rs stimulates it (Cardenas et al., 2010), implying that ER–mitochondrial uncoupling is involved. Indeed, loss of tethering mediated by PTPIP51 and VAPB inhibits basal autophagy, which, in turn, can be rescued with the introduction of artificial tethers (Gomez-Suaga et al., 2017). During autophagy, ER–mitochondria MCSs may concurrently function as sites of autophagosome formation (Hailey et al., 2010; Hamasaki et al., 2013; Li et al., 2017). In the early stages of cargo sequestration, the growing isolation membrane (IM) of the phagophore remains dynamically juxtaposed to the ER with the help of VAPB and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2)-focal adhesion kinase family interacting protein of 200 kD (FIP200; also known as RB1CC1) tethers, the Unc-51 like autophagy activating kinase 1 (ULK1) complex, SERCA pump and PI(3)P, which is produced by the kinase vacuolar protein sorting-associated protein 34 (VPS34; also known as PIK3C3) (Engedal et al., 2013; Hayashi-Nishino et al., 2009; Yl-Anttila et al., 2009; Zhao et al., 2017, 2018). Interestingly,

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VPS34 activity is regulated by its interaction with a $Ca^{2+}$-binding protein calmodulin in a $Ca^{2+}$-dependent manner, and clearance of PI(3)P from the IM coincides with its closure into a mature autophagosome, allowing subsequent fusion with endolysosomes to occur (Gulati et al., 2008; Zhao et al., 2017). Furthermore, increased $Ca^{2+}$ levels upon inhibition of SERCA or its activator vacuole membrane protein 1 (VMP1) have been shown to stabilize ER–IM contacts and inhibit autophagosome closure (Zhao et al., 2017). This suggests that a dynamic interplay between IP3Rs and SERCA activity is involved in the spatiotemporal control of autophagosome formation at ER–mitochondria MCSs. In addition, a recent study has implicated $Ca^{2+}$ release mediated by the late endosomal/lysosomal channel transient receptor potential mucolipin 1 (TRPML1; also known as MCOLN1) in IM formation through the PI(3)P-dependent mechanism described above (Scotto Rosato et al., 2019). Taken together, these findings point to a complex involvement of $Ca^{2+}$ and MCSs in autophagy initiation, which is likely to be expanded further in the future.

## $Ca^{2+}$ back and forth at ER–endolysosome MCSs

The endolysosomal system maintains its own unique relationships with the ER (Lee and Blackstone, 2020; Raiborg et al., 2015), and, akin to mitochondria, it stores a substantial amount of ER-derived $Ca^{2+}$ (Garrity et al., 2016; Kilpatrick et al., 2013). It is therefore not surprising that $Ca^{2+}$ efflux into the ER–endosome MCSs, or the peri-endolysosomal space, is important for endosome biology (Burgoyne et al., 2015; van der Kant and Neefjes, 2014). As endosomes progressively acquire late characteristics (i.e. become mature), they engage in more extensive interactions with the ER, and some can even sustain permanent contacts (Friedman et al., 2013). At the same time, as measured in pancreatic cells, in contrast to early endosomes, late endosomes and lysosomes harbor high levels of $Ca^{2+}$ (Albrecht et al., 2015; Yang et al., 2019). The exact mode(s) of $Ca^{2+}$ delivery to maturing endosomes is a matter of debate that has predominantly centered around the role of the endosomal pH (Yang et al., 2019). While it has recently been shown that the ER can siphon $Ca^{2+}$ into lysosomes irrespective of their acidity (Garrity et al., 2016), the molecular factors mediating lysosomal $Ca^{2+}$ (re)uptake remain unknown. Lysosomes have been observed to dwell around IP3R clusters at the ER membrane, and all three subtypes of IP3R channels were found capable of $Ca^{2+}$ delivery to these organelles (Atakpa et al., 2018; Garrity et al., 2016). This suggests that IP3Rs can create a local $Ca^{2+}$ microenvironment at ER–endosome MCSs, analogous to that at the ER–mitochondria interface. Considering the high availability of $Ca^{2+}$ in the vicinity of lysosomes following IP3R activation, any low-affinity $Ca^{2+}$ transporter or rectifying channel could be responsible for lysosomal $Ca^{2+}$ influx (Yang et al., 2019).

Once deposited into endolysosomes, $Ca^{2+}$ can also be released from these organelles by various channels that respond to different stimuli (see Box 1). Lysosomal $Ca^{2+}$ release, evoked experimentally through osmotic permeabilization, initiates prolonged $Ca^{2+}$ signals, underpinning the so-called ‘trigger hypothesis’ – small $Ca^{2+}$ spikes produced by lysosomal $Ca^{2+}$ channels, such as the two-pore channels (TPCs) or TRPML1, are postulated to trigger $Ca^{2+}$ release of greater amplitude from the ER, resulting in the establishment of both local $Ca^{2+}$ microenvironments and global $Ca^{2+}$ oscillations (Kilpatrick et al., 2013, 2016). Indeed, silencing of TPC1 or TPC2 reduces the extent of MCSs formed between the ER and either endosomes or lysosomes, respectively (Kilpatrick et al., 2017). In line with these findings, inhibition of $Ca^{2+}$ reuptake into the ER through the SERCA pump increases the amount and physical extent of these contacts (Zhao et al., 2017) Thus, the initial $Ca^{2+}$ spike from lysosomes could create conditions that induce MCS formation and/or enhance tether strength by stabilizing existing interactions on opposing membranes (Yang et al., 2019). In line with this, $Ca^{2+}$ release near lysosomes was found to activate a $K^+$ channel that reverses the lysosomal membrane potential, facilitating refilling of the lysosome with $Ca^{2+}$ (Wang et al., 2017). Additionally, a role for $Ca^{2+}$ in ER–endosome MCS formation suggests the presence of $Ca^{2+}$-binding tethers at contact sites where IP3Rs reside. For example, annexin A1 and its binding partner S100 $Ca^{2+}$-binding protein A11 (S100A11) can both bind $Ca^{2+}$ and have been shown to cooperate in order to bridge membranes *in vitro*. Together, these proteins form a tether for an ER–endosome MCS that controls epidermal growth factor (EGF) signaling (Fig. 4) (Eden et al., 2016, 2010; Gerke and Moss, 2002).

It is worth keeping in mind that MCSs constitute dynamic contacts between autonomous organelles that, once formed, can disengage (Fig. 4). This is exemplified by cholesterol sensors, such as Nieman–Pick intracellular cholesterol transporter 1 (NPC1), StAR related lipid transfer domain containing 3 (STARD3), or oxysterol-binding protein-related protein 1L (ORP1L; also known as OSBPL1A), which act as MCS tethers during transport of dietary cholesterol (LDL) from late endosomes to the ER for esterification (Alpy et al., 2013; Di Mattia et al., 2018; Hoglinger et al., 2019; Rocha et al., 2009) (Fig. 4). Intriguingly, cholesterol has been suggested to modulate detergent-resistant ER domains that house IP3Rs and their activator Sigma1R (Hayashi and Su, 2010; Weerth et al., 2007). Cholesterol-dependent membrane domains have also been implicated in proteasome-mediated degradation of activated IP3R clusters by a protein complex that includes RNF170 and

**Box 1. The endolysosomal system as a $Ca^{2+}$ reservoir**

Endosomes and lysosomes contain numerous $Ca^{2+}$-release channels (Feng and Yang, 2016), which can be engaged by a variety of stimuli. Among these, transient potential receptor mucolipins (TRPMLs) constitute the principal family of $Ca^{2+}$-release channels residing in endolysosomal membranes. Whereas TRPML2 and TRPML3 are found in early and recycling compartments (Lelouvier and Puertollano, 2011), TRPML1 predominantly localizes to lysosomes and responds to PI(3,5)P2, a phosphoinositide enriched on late compartments (Wallroth and Haucke, 2018). By binding to a polybasic domain in the N-terminus of TRPML1, PI(3,5)P2 may influence lysosomal $Ca^{2+}$ levels (Dong et al., 2010). TRPML isoforms have been shown to form functional homo- and hetero-multimers in cells (Curcio-Morelli et al., 2010), suggesting that they may act together (under different physiological conditions). Another channel family, the two-pore channels (TPCs), is activated in the presence of the $Ca^{2+}$-mobilizing messenger nicotinic acid adenine dinucleotide phosphate (NAADP) (Brailoiu et al., 2009; Galione, 2019). Although TPCs are best known for transporting monovalent cations ($Na^+$, $H^+$ and $K^+$), their activity is modulated by cytosolic $Ca^{2+}$ levels, as well as by PI(3,5)P2 and endolysosomal pH (Guo et al., 2017; Lagostena et al., 2017; Wang et al., 2012). Interestingly, TPC1 is enriched at ER–endosome MCSs (Kilpatrick et al., 2017), suggesting that it may regulate $Ca^{2+}$ exchange between these organelles. As TPC2 localizes predominantly on lysosomal membranes, it may perform a similar function there (Calcraft et al., 2009). Last, but not least, P2X4 channels are gated by ATP, which is highly abundant in lysosomes (Cao et al., 2015). Curiously, $Ca^{2+}$ release from these channels appears to be inhibited by a high luminal pH (Huang et al., 2014). These channels could therefore be involved in homotypic lysosomal fusion in order to maintain a healthy proteolytic compartment. Considering that the pH of the lysosome is linked to its spatial positioning in the cell (Johnson et al., 2016), these channels could also allow $Ca^{2+}$ influx in the more pH-neutral, peripherally located lysosomes.

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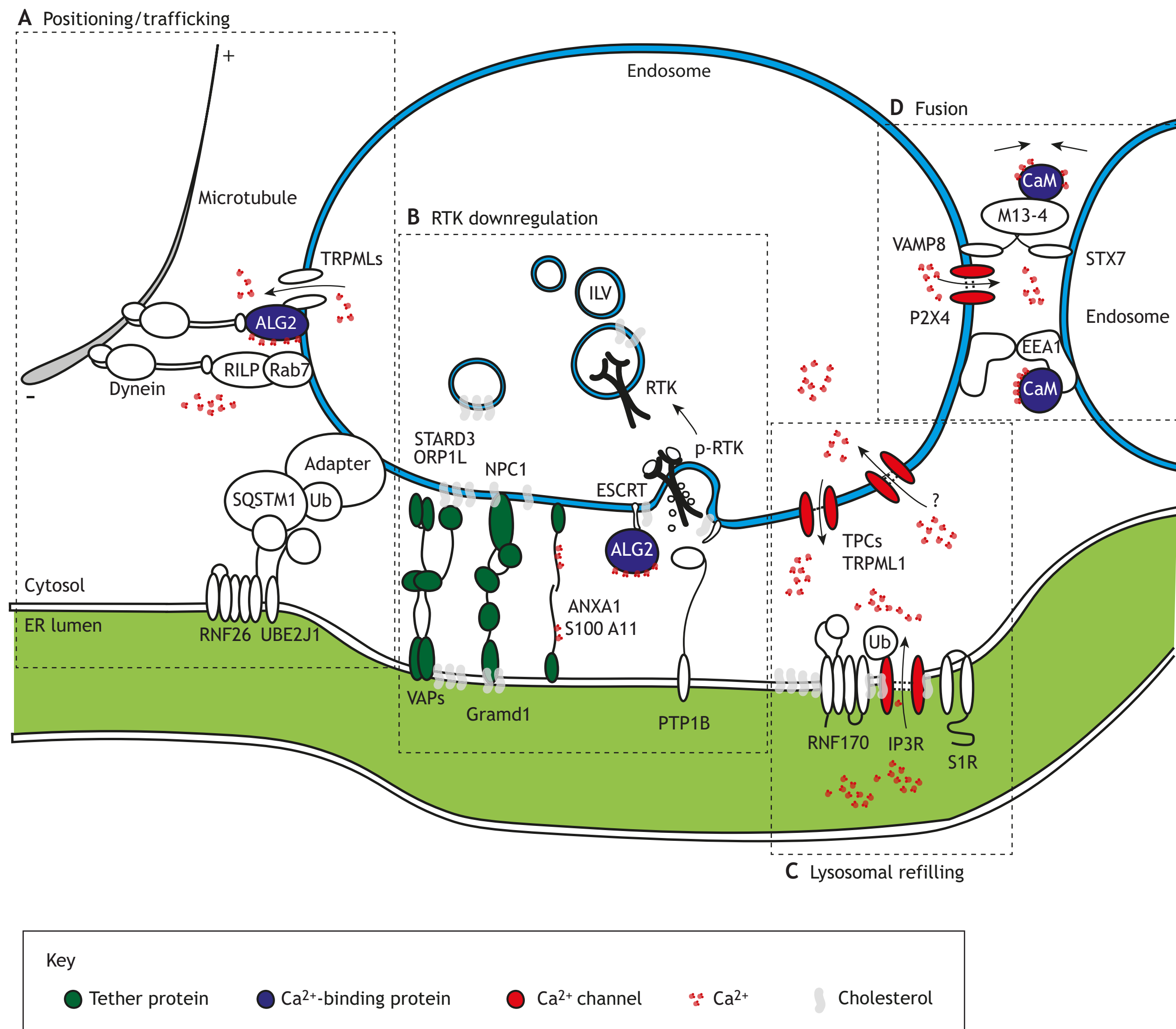


**Fig. 4. $Ca^{2+}$ at the ER–endolysosome MCSs regulates cargo trafficking, endosomal fusion and lysosomal refilling.** (A) Positioning and trafficking. In the course of their maturation, endosomes travel into the cell interior (towards the minus-end of microtubules); this requires active transport that is mediated by the cholesterol-sensitive RILP–dynein complex, or the $Ca^{2+}$-responsive ALG2–dynein complex. Once in the perinuclear region, endosomes can interact with the positioning complex, comprising the ER-membrane-resident RNF26 protein and its substrate SQSTM1. (B) RTK downregulation. At these contact sites, cholesterol exchange occurs via the ORP1L–STARD3 and/or the NPC1–GRAMD1 axes. Under conditions of ligand stimulation, contact sites are formed between the ER and endosomes that carry activated receptor tyrosine kinases (RTKs), such as phosphorylated epidermal growth factor receptor (p-EGFR) to support efficient receptor downregulation. These MCSs rely on the $Ca^{2+}$-dependent annexin A1 (ANXA1) tether that works in conjunction with its ligand S100A11 and provide a platform for the ER-associated PTP1B to dephosphorylate and thus inactivate its substrate EGFR. PTP1B and ALG2 also stimulate the ESCRT machinery towards cholesterol-dependent ILV formation, terminating RTK degradation. (C) Lysosomal refilling. Lysosomal $Ca^{2+}$ release through two-pore channels (TPCs) or mucolipin transient receptor potential (TRPML) channels evokes $Ca^{2+}$ efflux from the ER by activating the IP3R–SigmaR1 complex, which is negatively regulated through ubiquitin-mediated degradation induced by RNF170 and associated proteins that localize to cholesterol-rich ER subdomains. The resulting local $Ca^{2+}$ microenvironment influences the formation of the ER–endosome/lysosome MCSs. Transporter(s) needed for lysosomal $Ca^{2+}$ filling have not yet been established. (D) Fusion. Peri-endosomal $Ca^{2+}$ released through ATP- and pH-dependent P2X4 channels may regulate fusion between different types of endosomes that is mediated by calmodulin (CaM), Munc13-4 (M13-4) and the endosomal soluble NSF attachment receptor (SNARE) proteins syntaxin 7 (STX7) and VAMP8.

Erlin1–Erlin2 (Browman et al., 2006; Pearce et al., 2009). Therefore, cholesterol sensing in late endosomes and/or the ER membrane may set the stage for both activation and degradation of IP3Rs, thus regulating $Ca^{2+}$ release into the MCS and (un)tethering of the participating organelles. In addition to cholesterol-associated formation of ER-endosomes MCSs, ubiquitin-mediated events, such as those regulated by the ER-embedded ubiquitin ligase ring finger-containing protein 26 (RNF26), ubiquitin-conjugating enzymes E2 J1 (UBE2J1) and sequestosome 1 (SQSTM1; also known as p62) complex (Cremer et al., 2020 preprint; Jongsma et al., 2016) can also support reversible connections between these organelles. Interestingly, recent proteomic findings suggest that RNF26 resides in a complex with various members of the ER $Ca^{2+}$ efflux machinery, including IP3R1 and SigmaR1 (Fenech et al., 2020). The observed dynamics of lysosomes at IP3R clusters parallel those of endolysosomes that reversibly associate with the ER via

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RNF26 (Atakpa et al., 2018; Jongsma et al., 2016). It is therefore possible that $Ca^{2+}$ release channels are coregulated with the recruitment of endosomes to $Ca^{2+}$-rich signaling hubs at the ER membrane. Although much remains to be uncovered with regard to the influence of $Ca^{2+}$ signaling on ER–endosome MCS formation, a wealth of knowledge on how $Ca^{2+}$ contributes to the physiology of the endolysosomal system has been gained in recent years, as discussed below.

## $Ca^{2+}$ in the regulation of endosome biology

The $Ca^{2+}$ microenvironment in the endosome provides the basis for key processes regulating the function of these organelles, as evident from the documented connections between lysosomal $Ca^{2+}$ storage defects and neurodegenerative diseases (reviewed in Feng and Yang, 2016). How $Ca^{2+}$ modulates dynamic events that are pivotal for endosome biology, including acquisition of multivesicular body (MVB) architecture along the endosomal maturation route, is discussed in the following sections and summarized in Fig. 4.

At multiple stages of endocytosis, vesicles of the endosomal system fuse to mix their constituents (Balderhaar and Ungermann, 2013). For instance, early endosomal antigen 1 (EEA1) is recruited to Rab5-positive endosomes in a $Ca^{2+}$/calmodulin-dependent manner, where it mediates apposition with the target membrane (Lawe et al., 2003; Murray et al., 2016). As endosomes continue on their maturation route, they become MVBs (Scott et al., 2014), and $Ca^{2+}$ signaling facilitates this process in a number of ways. For instance, loss of late endosomal $Ca^{2+}$ has been linked to lysosomal storage diseases, including the Niemann–Pick type 1 disease (Lloyd-Evans et al., 2008). Additionally, $Ca^{2+}$ influences ligand-mediated sorting of receptor tyrosine kinases (RTKs), such as the EGF receptor (EGFR) for their lysosomal degradation. The signaling of phosphorylated EGFR can be terminated by the ER-localized phosphatase PTP1B, which encounters these endocytosed receptors at ER-endosome MCSs that are tethered by $Ca^{2+}$-responsive annexin A1 (Eden et al., 2012, 2016). The same dephosphorylation event then facilitates the commitment of EGFR for degradation. It has been suggested that $Ca^{2+}$ release through NAADP-activated TPC1 channels promotes the formation of ER–endosome contacts, thus enhancing the accessibility of the receptor substrate to PTP1B (Kilpatrick et al., 2017). Subsequently, $Ca^{2+}$ comes into play once again through the $Ca^{2+}$-binding protein asparagine-linked glycosylation protein 2 (ALG2), which controls cholesterol-, Alix (PDCD6IP)- and endosomal sorting complexes required for transport (ESCRT)-mediated membrane deformation during the incorporation of cargo into intralumenal vesicles (ILVs) (Maki et al., 2016; Missotten et al., 1999; Scheffer et al., 2014; Sun et al., 2015). Illustrating their respective significance, inhibition of PTP1B, annexin A1, TPC1 and Alix has been shown to prolong potentially hazardous EGFR signaling (Eden et al., 2016, 2010; Kilpatrick et al., 2017; Sun et al., 2015). Similarly, vascular endothelial growth factor (VEGF) signaling is prolonged as a consequence of either NAADP or TCP2 inhibition, suggesting that $Ca^{2+}$ is a general regulator of RTK signaling termination (Favia et al., 2014).

To enhance fusion of endosomal cargo carriers with the proteolytic compartment, the bulk of incoming traffic is targeted to the perinuclear vesicle cloud (Jongsma et al., 2016). Transport to the perinuclear area is regulated by several routes (reviewed in Cabukusta and Neefjes, 2018), including the one controlled by the GTPase Rab7, Rab-interacting lysosomal protein (RILP) and the dynein motor (Wijdeven et al., 2016). An alternate minus-end-directed route involves the PI(3,5)P2-activated $Ca^{2+}$ channel TRPML1 and $Ca^{2+}$-responsive endosomal dynein adaptor protein ALG2 (Li et al., 2016; Vergarajauregui et al., 2009). Subsequently, the fusion of endosomes with lysosomes is mediated by the homotypic fusion and protein sorting (HOPS) complex and the $Ca^{2+}$-dependent interaction of Munc13-4 (also known as UNC13D) with syntaxin 7 and vesicle-associated membrane protein 8 (VAMP8), which are present on opposing membranes (He et al., 2016). In the presence of ATP, lysosomal P2X4 (also known as P2RX4) channels release $Ca^{2+}$ and facilitates calmodulin-dependent activation of Munc13-4, which in turn connects syntaxin 7 and VAMP8 (Cao et al., 2015; Herbst et al., 2014). Munc13-4 is additionally known to mediate homotypic fusion and secretion of lysosome-like granules in immune cells, emphasizing a role for $Ca^{2+}$ in vesicle fusion (Ramadass and Catz, 2016). While important contributions of $Ca^{2+}$ to endosomal transport and fusion have been elucidated, the relevance of ER–endosome MCSs in this context remains to be investigated.

Most endocytic (as well as autophagic) cargoes ultimately find their way into the lumen of the lysosome, where their degradation takes place. To ensure the fitness of lysosomes in subsequent rounds of proteolysis, these organelles are continuously remodeled through regulated fission and fusion events, which involve the interplay between two lysosomal $Ca^{2+}$ channels, P2X4 and TRPML1. Localized lumen alkalization has been suggested to activate P2X4 channels (see Box 1) and drive calmodulin-mediated homotypic fusion in a $Ca^{2+}$-dependent manner (Cao et al., 2015; Huang et al., 2014). Furthermore, P4X2 overexpression induces enlargement of the lysosome, which could be suppressed by overexpressing the other $Ca^{2+}$ channel, TRPML1, but not TPC2; intriguingly, this also depends on $Ca^{2+}$ binding by calmodulin (Cao et al., 2017). Based on these findings, the Cao et al. proposed that TRPML1 controls lysosomal size through fission (Cao et al., 2017). In contrast to P2X4, TRPML1 is inhibited by alkaline pH (Dong et al., 2008). Thus, calmodulin may recruit different effectors, depending on the lysosomal context, to regulate membrane dynamics of these organelles. Taken together, the observations discussed above showcase the diversity of contributions made by $Ca^{2+}$ to the integrity and function of the endolysosomal system and open new avenues for future research.

## Conclusions and future directions

In conclusion, as a widely conserved and ancient signaling entity, $Ca^{2+}$ harbors enormous influence over the biochemical and physiological aspects of cellular life. For these very reasons, $Ca^{2+}$ is held inside organellar stores, allowing spatially and temporally regulated release. As discussed in this Review, accomplishing this feat requires the concerted action of multiple organelles. At the interface between the ER and PM, $Ca^{2+}$ enters the cell, where it engages powerful feedback mechanisms tasked with ensuring $Ca^{2+}$ homeostasis. Once in the ER, $Ca^{2+}$ facilitates a variety of basic cellular needs, including protein folding and degradation, and, as it flows through the ER lumen and out into various MCSs, $Ca^{2+}$ instigates dynamic responses from the participating organelles with wide-ranging implications for cellular metabolism and responsiveness to external stimuli. Through negative feedback on its own release, progressive signaling into the center of the cell, fast $Ca^{2+}$ buffering in the cytoplasm and re-uptake into the ER, cells show a highly dynamic and tightly regulated $Ca^{2+}$ signaling cadence. The resulting ebb and flow of $Ca^{2+}$ produces a broad range of oscillations that can be regarded as the beat to which the organelles of the cell dance. While substantial progress in uncovering the choreography of the influence of $Ca^{2+}$ over the

behavior and function of cellular organelles has been made, many questions remain open. These include the nature of molecular mechanisms controlling ER flow (and the involvement of $Ca^{2+}$ therein), as well as components responsible for $Ca^{2+}$ entry into vesicles of the endolysosomal system. With the development of novel tools, such as pH-resistant $Ca^{2+}$ sensors, increasingly more powerful (live) imaging tools and high-throughput genetic screening options, these and other questions may soon find their answers.

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