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COMMENTARY

Small regulators, major consequences – Ca^{2+} and cholesterol at the endosome–ER interface

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ABSTRACT

The ER is the largest cellular compartment and a major storage site for lipids and ions. In recent years, much attention has focused on contacts between the ER and other organelles, and one particularly intimate relationship is that between the ER and the endosomal system. ER–endosome contacts intensify when endosomes mature, and the ER participates in endosomal processes, such as the termination of surface receptor signaling, multi-vesicular body formation, and transport and fusion events. Cholesterol and Ca^{2+} are transferred between the ER and endosomes, possibly acting as messengers for ER–endosome crosstalk. Here, we summarize different types of ER–endosomal communication and discuss membrane contact sites that might facilitate this crosstalk. We review the protein pairs that interact at the ER–endosome interface and find that many of these have a role in cholesterol exchange. We also summarize Ca^{2+} exchange between the ER and endosomes, and hypothesize that ER–endosome contacts integrate several cellular functions to guide endosomal maturation. We post the hypothesis that failure in ER–endosome contacts is an unrecognized but important contributor to diseases, such as Niemann–Pick type C disease, Alzheimer's disease and amyotrophic lateral sclerosis.

KEY WORDS: Ca^{2+}, Cholesterol, Endoplasmic reticulum, Endosome, Lysosome, Membrane contact site

Introduction

From its first description in 1945, the endoplasmic reticulum (ER) has been intensely studied. It represents an interconnected network of tubules and cisternae that occupies the entire cytosolic space. Rough ER (ER lined by ribosomes) defines sites of protein translation, whereas the smooth ER is considered a site of lipid production and a storage area for lipids and ions. The nuclear envelope can be considered a third ER 'subdomain'. The ER is by far the largest intracellular organelle; it contains ~70% of cellular lipids and contributes to the generation of other organelles such as the Golgi complex. Through specific interactions, ER-resident proteins contact their interaction partners, which are located on other organelles, generating membrane contact sites (MCSs). MCSs are sites of close apposition between two organelles, classically defined as having a 10 to 30 nm space between their membranes. For comparison, the size of a lipid bilayer is ~10 nm, whereas a small protein such as GFP is ~5 nm. MCSs can be considered intracellular synapses at which molecular information can be exchanged within a confined space. MCSs between ER and multiple organelles have been reported, including the plasma membrane, mitochondria, Golgi and endosomes (Elbaz and Schuldiner, 2011). The ER–endosome relationship appears to be especially tight and it has been estimated that, in some cells, over 99% of all late endosomes are in contact with ER membranes (Friedman et al., 2013).

In this Commentary, we will discuss processes that are known to occur at the ER–endosome interface. We introduce the concept that the ER regulates many steps in endosome biology by initiating transient contact sites. We summarize the known protein pairs that bridge ER–endosome membranes and find that many of these are involved in cholesterol transfer. We then outline cellular cholesterol flow and show how ER–endosome contact sites allow endocytosed cholesterol to arrive to the ER for storage. In addition to cholesterol exchange, we also summarize recent data on Ca^{2+} exchange between the ER and endosomes. Endosomes can release Ca^{2+} to initiate signaling cascades, but also store and buffer Ca^{2+} released by the ER. Endosomes also require Ca^{2+} at several maturation steps, and uptake of Ca^{2+} by endosomes is likely regulated at ER–endosome contacts. We discuss how regulation of endosomal maturation by the ER extends beyond Ca^{2+} regulation and is in control of processes such as growth factor signaling and endosomal transport. We conclude by discussing diseases and disease mechanisms that result from mutations in proteins that function at ER–endosome contacts. From this, the view emerges that endosomes are not independent entities but embedded in a large organellar network with the ER as the control center.

Endocytosis

Endocytosis is a major process in cellular homeostasis, which allows extracellular signals and content to enter the cell. Endocytosed material, such as signaling receptors, low-density lipoprotein (LDL) particles and other nutrients, are delivered to the acidic lysosome where they are degraded into essential cellular building blocks such as amino acids and lipids (reviewed in Huotari and Helenius, 2011). Bacteria and viruses also use this system to enter cells (Fairn and Grinstein, 2012). The molecular basis for many steps in the endosomal pathway has been unraveled over the past decades, including the control of transport, maturation, fusion and multivesicular body formation. Endosomal RAB GTPases are major controllers of these steps (Box 1) and the assumption for many years was that endosomes act autonomously with only little involvement from other compartments. However, during recent years, a crucial role for the ER in shaping endosomal biology has become apparent (Fig. 1). This includes a control of endosomal processes by the ER, such as in surface receptor signaling (Eden et al., 2010), in

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Box 1. Rab GTPases and endosome identity

Rab GTPases are a large family (>60 members in humans) of small GTPases. They function as molecular switches that are turned 'on' by GTP binding and are 'off' when bound to GDP (Stenmark, 2009). Rab GTPases define organelle identity and are central players in organelle dynamics. When activated, Rab GTPases can recruit effectors, such as motor-, tether-, fission- and sorting-protein complexes to target membranes. After internalization from the plasma membrane, endocytic carriers fuse to form early endosomes. Early endosomes are a major sorting station for endocytosed cargo and contain several Rab GTPases (Jovic et al., 2010). Rab4 and Rab5 are the best studied members; Rab4 links early endosomes to Rab11-positive recycling endosomes and facilitates the retrieval of endocytosed material to the plasma membrane (reviewed in Jovic et al., 2010). Rab5 aids in the maturation, fusion and transport of early endosomes and initiates the recruitment of Rab7 (a late endosomal GTPase) to maturing endosomes (reviewed in Huotari and Helenius, 2011). Late endosomes also contain Rab9, which is involved in cargo transport between late endosomes and the trans-Golgi network (Huotari and Helenius, 2011). Rab7 remains bound to most stages of late endosomes, lysosomes and lysosome-related organelles to drive transport and fusion.

lipid transfer (Du et al., 2011) and endosomal transport (Rocha et al., 2009). Proteins that are present at ER–endosome contacts also regulate intraluminal vesicle (ILV) formation (Kobuna et al., 2010; Du et al., 2012) and late endosomal fusion (van der Kant et al., 2013b). Although it is likely that these processes are regulated at ER–endosome MCSs, it is difficult to visualize and quantify these contact sites in living cells because the distance between the membranes of 30 nm is below the resolution of normal light microscopy (>240 nm). Nevertheless, using a combination of electron microscopy and light microscopy, it has been estimated that half of the early endosomes are in contact with the ER. During endosome maturation, this contact intensifies and almost all late endosomes are in close proximity to the ER (Friedman et al., 2013). The ER–endosome contacts are not the result of random collisions but are controlled by specific ER–endosome-tethering complexes, as will be specified below.

Protein pairs at the ER–endosome interface

The endosomal oxysterol-binding protein (OSBP)-related protein 1L (ORP1L) was the first factor shown to induce ER–endosome MCSs by contacting the ER protein vesicle-associated membrane protein (VAMP)-associated protein A (VAP-A) (Rocha et al., 2009) (Fig. 1). VAP-A is more generally involved in interactions between the ER and other organelles as it interacts with proteins containing a FFAT motif [two phenylalanines (FF) in an acidic tract] that are present at the surface of many organelles (Loewen et al., 2003; Loewen and Levine, 2005; Elbaz and Schuldiner, 2011). FFAT motifs bind to a positively charged patch on the surface of VAP (Kaiser et al., 2005). Although the conventional FFAT consensus sequence is defined as E**FF**DAxE, small variations are possible, as illustrated by the FFAT motif sequence E**FY**DALS in ORP1L (Rocha et al., 2009). Other proteins with a non-canonical FFAT motif (Q**FY**SPPE) are metastatic lymph node 64 [MLN64, also known as steroidogenic acute regulatory protein (StAR)-related lipid transfer domain protein 3 (STARD3)], a transmembrane protein present on a subpopulation of late endosomes (van der Kant et al., 2013a), and a shorter analogue MLN64 N-terminal homologue [MENTHO, also known as STARD3 N-terminal like protein (STARD3NL)] and both interact with VAP-A (Alpy et al., 2013) (Fig. 1). VAP-A is not the only ER protein that interacts with endosomal proteins. Oxysterol-binding protein-related protein 5 (ORP5), an ER-resident transmembrane protein related to ORP1L, contacts the late endosomal cholesterol transporter Niemann-Pick type C1 (NPC1) (Du et al., 2011) and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs, also known as HGS) that is located on endosomes (Pridgeon et al., 2009; Du et al., 2012). Furthermore, the ER protein-tyrosine phosphatase 1B (PTP1B) interacts with epidermal growth factor receptor (EGFR) as well as with the granulocyte colony-stimulating factor receptor (G-CSFR, also known as CSF3R) – the later involving Prdx4 (Eden et al., 2010; Palande et al., 2011). Apart from the interaction between PTP1B and EGFR or G-CSFR, the above-mentioned interactions (ORP1L- or MLN64 and VAP-A, and ORP5 and NPC1) all involve cholesterol.

Cholesterol exchange at the junction – fueling the relationship

As most interactions between the ER and endosomal proteins involve cholesterol-binding proteins, it is plausible that cholesterol controls ER–endosome interactions and/or their maintenance. Cholesterol might act as a second messenger for contacts between the ER and endosomes, and/or ER–endosome contact sites could act as confined regions for the exchange of cholesterol and possibly of other substrates between these two compartments. Indeed, the existence of contact sites is linked with the flow of cholesterol through cellular compartments. There are two ways for cells to acquire cholesterol; *de novo* cholesterol synthesis and endocytosis of circulating cholesterol-containing particles (reviewed in Ikonen, 2008). Most cells internalize LDL particles (packaged by the liver) through the plasma membrane LDL receptor (LDLR) (Fig. 2). These particles are subsequently transported to acidic late endosomes that can contain over half of the total cholesterol content in the endocytic pathway (Möbius et al., 2003). Here, the LDL particles are hydrolyzed by acid lipases into unesterified cholesterol for cellular distribution. Although cholesterol is processed and released in late (multivesicular) endosomes, it subsequently has to be transported to other compartments, such as the ER, the plasma membrane (PM) and mitochondria (Neufeld et al., 1996; Lange et al., 1997; Zhang et al., 2002). Given that the ER contacts all these compartments, it is plausible that cholesterol from late endosomes is distributed to these other organelles via the ER. However, cholesterol from late endosomes first has to reach the ER.

A substantial amount (~30%) of late endosomal cholesterol is directly transported from late endosomes to the ER (Neufeld et al., 1996). As cholesterol is a hydrophobic compound, this cannot be achieved by simple diffusion through the water phase of the cytosol. Cytosolic cholesterol-transporting proteins could distribute cholesterol to many sites by diffusion, but a more directed manner of cholesterol transport occurs through MCSs. Interactions between ORP5 and NPC1 at the ER–late-endosome interface facilitate transfer of cholesterol from the late endosome to the ER (Du et al., 2011). In a sequence of events, a soluble cholesterol carrier in the lumen of the endosome (NPC2) delivers luminal cholesterol to the transmembrane protein NPC1. NPC1 facilitates the movement of cholesterol to the cytosolic leaflet of

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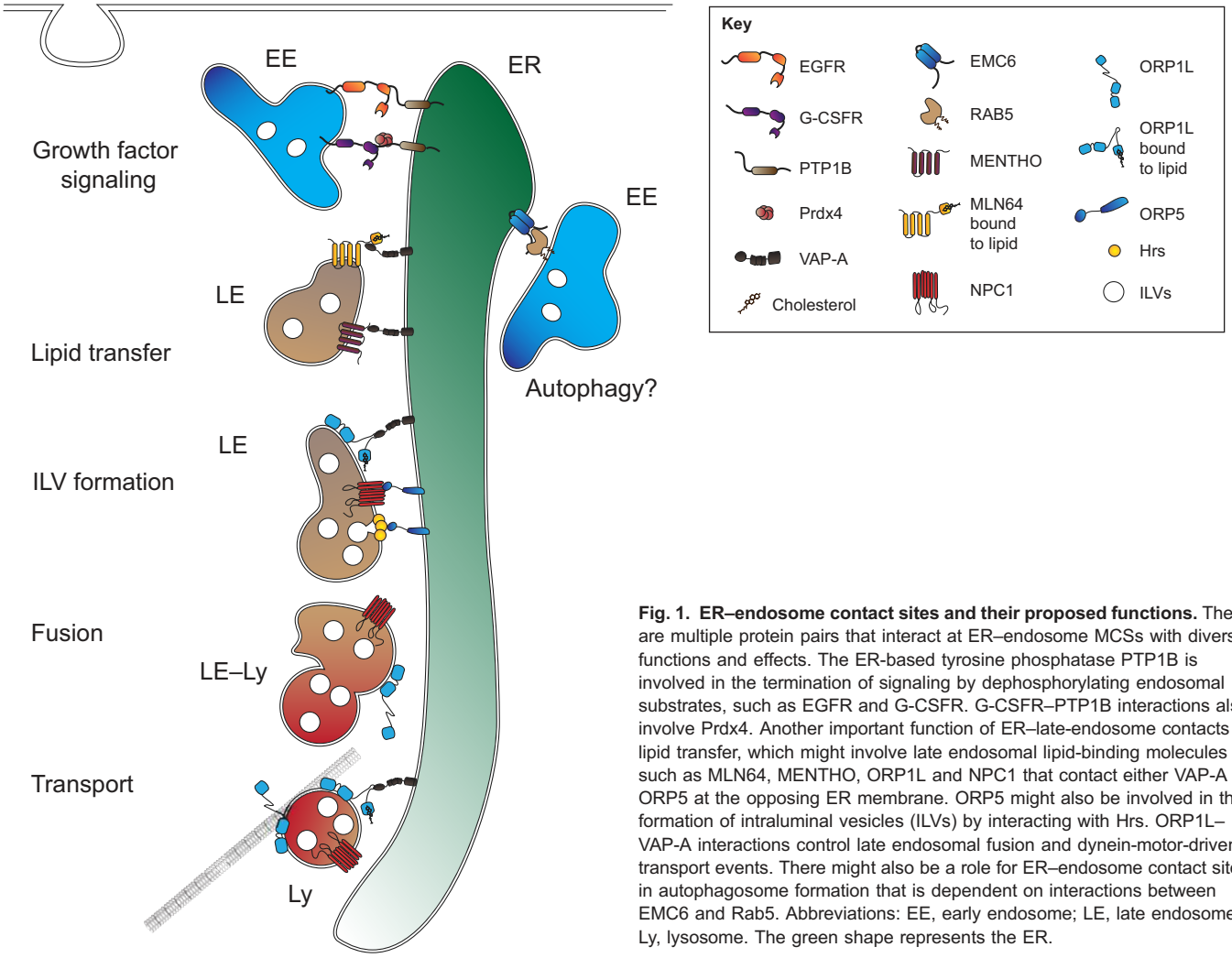

Fig. 1. ER–endosome contact sites and their proposed functions. There are multiple protein pairs that interact at ER–endosome MCSs with diverse functions and effects. The ER-based tyrosine phosphatase PTP1B is involved in the termination of signaling by dephosphorylating endosomal substrates, such as EGFR and G-CSFR. G-CSFR–PTP1B interactions also involve Prdx4. Another important function of ER–late-endosome contacts is lipid transfer, which might involve late endosomal lipid-binding molecules such as MLN64, MENTHO, ORP1L and NPC1 that contact either VAP-A or ORP5 at the opposing ER membrane. ORP5 might also be involved in the formation of intraluminal vesicles (ILVs) by interacting with Hrs. ORP1L–VAP-A interactions control late endosomal fusion and dynein-motor-driven transport events. There might also be a role for ER–endosome contact sites in autophagosome formation that is dependent on interactions between EMC6 and Rab5. Abbreviations: EE, early endosome; LE, late endosome; Ly, lysosome. The green shape represents the ER.

the late endosome membrane. At this site of the endosomal membrane, cholesterol is bound by ORP5 (a transmembrane ER protein) and inserted into the ER membrane (Subramanian and Balch, 2008; Du et al., 2011) (Fig. 2). These steps of cholesterol trafficking are important for cellular homeostasis because mutations in NPC1 and NPC2 cause Niemann Pick type C disease. In this neurodegenerative disease, cholesterol fails to reach the ER and remains trapped in late endosomes (Mukherjee and Maxfield, 2004; Lloyd-Evans et al., 2008). It is not known whether ORP5–NPC1 interactions are sufficient to initiate interactions between the ER and late endosomes, or instead shuttle cholesterol after the sites are formed. Contact sites can be initiated by the ORP family member ORP1L that is present on the same late endosomes as is NPC1 (van der Kant et al., 2013a). ORP1L promotes late endosomal contacts with the ER when cholesterol levels at the cytosolic leaflet of the endosome are low (Rocha et al., 2009). Under conditions where late endosomes have a high cholesterol content, ORP1L undergoes a conformational change that renders it unable to interact with VAP-A, thereby disrupting late-endosome–ER contact sites. As ORP1L-dependent contacts are only formed under low cholesterol conditions, this suggests that ORP1L does not transfer cholesterol directly (Rocha et al., 2009). One explanation for this apparent paradox could be that ORP1L initiates ER–late-endosome contacts only when cholesterol is bound to other proteins (such as NPC1). Cholesterol binding to NPC1 would limit the amount of free (protein unbound) cholesterol in the cytosolic phase of late endosomal membranes that is available for binding by ORP1L. ORP1L could then sense this to initiate contacts with the ER and allow cholesterol exchange through ORP5 and NPC1. Before cholesterol enters into endosomes that contain NPC1 and ORP1L, it is found in a distinct MLN64-positive (but ORP1L and NPC1 negative) subpopulation of late endosomes that also contains the ATP-binding cassette sub-family A member 3 (ABCA3) (van der Kant et al., 2013a). In a similar way to ORP1L, MLN64 can also initiate ER–late-endosomes contacts by binding to VAP-A through its FFAT motif (Alpy et al., 2013). MLN64 contains a steroidogenic acute regulatory (StAR) domain that could be involved in the shuttling of cholesterol between membranes (Alpy and Tomasetto, 2005). However, MLN64 is not likely to transfer cholesterol to the ER, as MLN64 overexpression does not enhance cholesterol esterification in the ER (Liapis et al., 2012), but instead increases sterol deposition in late endosomes (Hölttä-Vuori et al., 2005). Therefore, in a similar manner to ORP1L, MLN64 might only be involved in the initiation of contact between the ER and late endosomes, while leaving active cholesterol transfer to other proteins.

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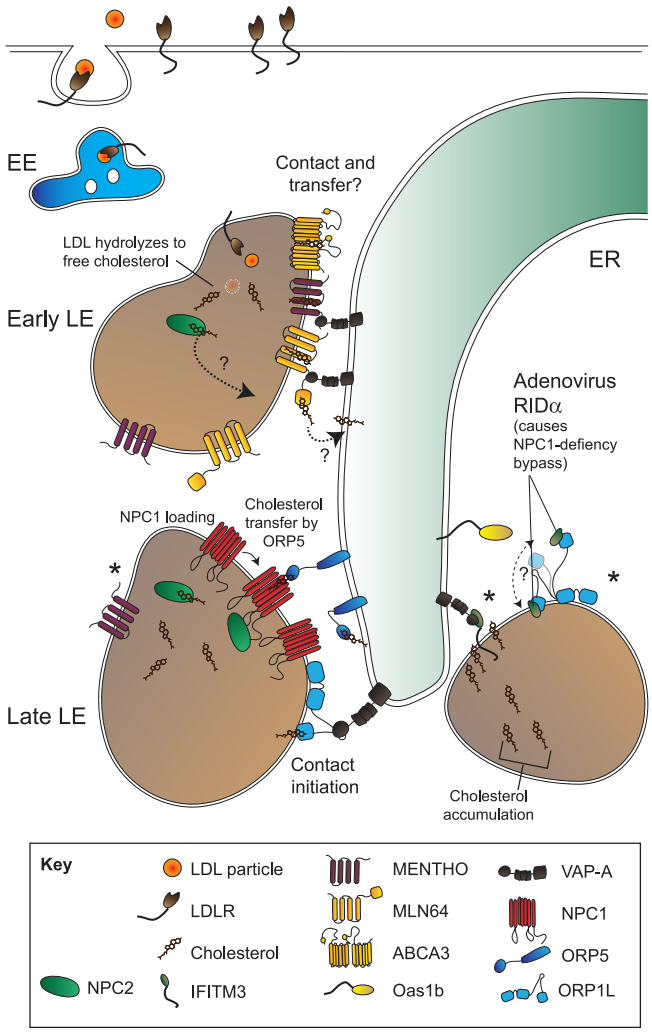

Fig. 2. Cholesterol transfer at the ER–endosomal interface. Cholesterol that is packaged in LDL particles enters cells by binding to LDL receptors (LDLRs) that are subsequently transported to late endosomes. LDL particles are then hydrolyzed and free cholesterol is released. Cholesterol first encounters an 'early' late endosomal (LE) compartment marked by MLN64 and ABCA3 and later enters a 'late' LE compartment marked by NPC1 and ORP1L. As yet, only NPC2, NPC1 and ORP5 have been implicated in direct cholesterol transfer to the ER. NPC2 binds free luminal cholesterol and passes it on to NPC1. NPC1 is involved in the transfer of the cholesterol to the cytosolic phase of the endosomal membrane where it is bound by ORP5 and transferred to the ER. ER–endosomal cholesterol transfer can be manipulated by viral and host anti-viral proteins (shown on the bottom right). The adenovirus RIDα protein and the host antiviral protein Oas1b interact with ORP1L to alter cholesterol trafficking. RIDα promotes cholesterol transport from the LE to the ER even in the absence of functional NPC1 (NPC1 deficiency bypass). The host antiviral protein IFTIM3 interacts with VAP-A and induces cholesterol accumulation. For proteins indicated with asterisk, it is not known whether these localize to a specific endosomal subpopulation or can be found on both the ORP1L/NPC1-positive LE subpopulation, as well as on the MLN64/ABCA3-positive subpopulation. The green shape represents the ER.

Neither depletion of ORP1L nor MLN64 results in an accumulation of cholesterol in late endosomes (Rocha et al., 2009; Hölttä-Vuori et al., 2005), which suggests that there are multiple alternative stages of endosomes from which cholesterol can be exported. These could either be spatially separated 'safeguards' that allow for multiple cholesterol exit sites or they could reflect functionally different contacts sites that serve to transport cholesterol to different final destinations. Alternatively, ORP1L- and MLN64-induced contacts might be involved in a reverse cholesterol transport from the ER to late endosomes, or might in fact be dispensable for cholesterol transfer and instead required for the regulation of endosomal transport and/or Ca^{2+} transfer.

Although the role for the proteins described above in ER–late-endosome MCSs is becoming clearer, it is likely that additional proteins are required to regulate additional processes that occur in MCSs. These probably include proteins, such as the above-mentioned VAP-A interactor MENTHO (Alpy et al., 2013) and the ORP5-interacting factor Hrs (Du et al., 2012). Other proteins, such as the adenovirus RIDα (receptor internalization and degradation) (Cianciola et al., 2013), the antiviral proteins interferon-induced transmembrane protein 3 (IFITM3) (Amini-Bavil-Olyaee et al., 2013) and oligoadenylate synthetase 1b (Oas1b) (Courtney et al., 2012) (Fig. 2) moderate cholesterol transfer and endosomal function at the ER–endosome interface, but exactly how is not known. Interestingly, expression of RIDα rescues the export of late endosomal cholesterol to the ER in NPC1-deficient cells (Cianciola et al., 2013). Finally, it is unclear how ORP1L-containing late endosomes are separated from those that contain MLN64. This separation suggests that there is some form of communication between these two cholesterol-sensing systems.

Ca^{2+} at the ER–endosome interface

Intracellular Ca^{2+} storage

The ER is also a major storage site for Ca^{2+} and an important regulator of cellular Ca^{2+} signaling. Ca^{2+} exchange at mitochondrial–ER and plasma-membrane–ER contact sites has been extensively described (reviewed in Elbaz and Schuldiner, 2011), but Ca^{2+} crosstalk between the ER and endosomes is a relatively new concept. Cellular calcium ions act as second messengers in many physiological processes, such as growth factor signaling, neurotransmission, vesicle fusion, G-protein-coupled receptor signaling and heart contraction (Berridge et al., 2000). Consequently, cellular Ca^{2+} levels are tightly regulated. Free cytosolic Ca^{2+} concentrations are low (typically 100 nM), which is 10,000-fold lower than in the extracellular milieu (1 mM) (Fig. 3). Intracellular Ca^{2+} levels can increase 10- to 100-fold during various cellular functions when Ca^{2+} channels in the plasma membrane and ER open. Early endocytic carriers that are derived from membranes that engulf extracellular fluid likely contain Ca^{2+} in similar concentrations as in the extracellular environment (Patel and Docampo, 2010). Ca^{2+} is, however, quickly released from endosomes and reduced to a concentration of only 3–40 µM within 20 minutes (Gerasimenko et al., 1998; Sherwood et al., 2007). The transient receptor potential cation channel, mucolipin subfamily, member 3 (TRPML3) might mediate this Ca^{2+} leak from endosomes (Lelouvier and Puertollano, 2011). Ca^{2+} concentrations in late endosomes or lysosomes have been reported to be significantly higher (~500 µM) and in the same range as in the ER (around 60–400 µM) (Christensen et al., 2002; Lloyd-Evans et al., 2008). This implies that lysosomes either concentrate Ca^{2+} or can absorb Ca^{2+} from other sources such as the ER. The fact that lysosomal Ca^{2+} (~500 µM) concentrations are much higher than cytosolic levels (~100 nM) also indicates that endosomal compartments might actively participate in cellular Ca^{2+} signaling.

Ca^{2+} signaling by endosomes

Indeed, cytosolic Ca^{2+} levels significantly rise during lysosomal permeabilization (Kilpatrick et al., 2013). The initial rise directly

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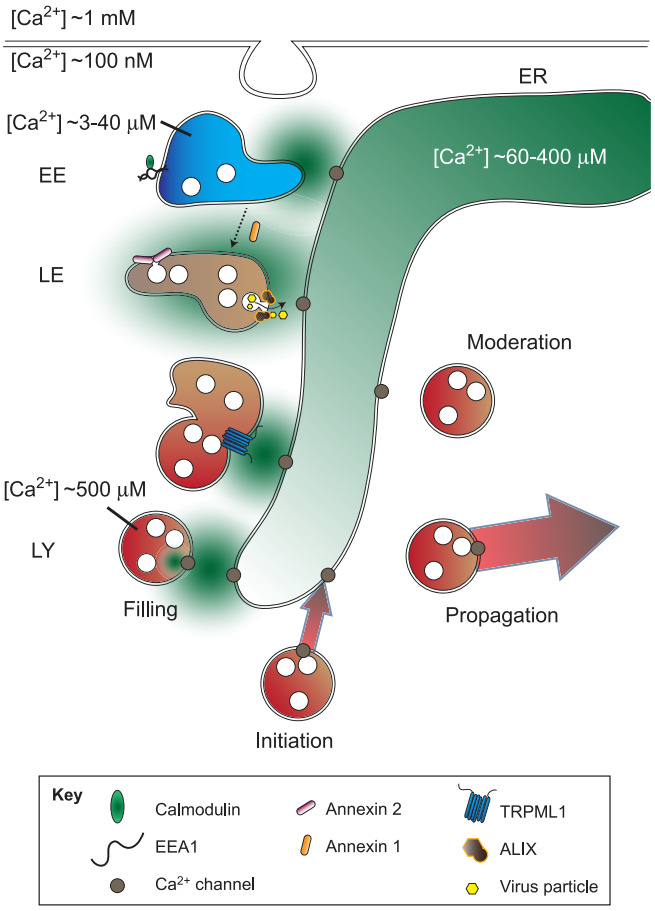

Fig. 3. Crosstalk between Ca^{2+} signaling in the ER and endosomes. Ca^{2+} release is a major second messenger in cells. Ca^{2+} levels in the cytosol are kept low, whereas organelles, such as the ER and lysosomes, serve as Ca^{2+} storage sites. The figures indicate the Ca^{2+} concentrations in early endosomes (EE), late endosomes (LE), lysosomes (Ly) as well as the extracellular and cytosolic calcium concentrations. EEA1 and calmodulin act together to initiate early endosomal fusion, a step that requires Ca^{2+}. Ca^{2+} release (green 'cloud') by Ca^{2+} channels at ER–endosome contact sites in the ER could be a potential source for the Ca^{2+} that is required for this fusion. Multivesicular endosome biogenesis and invagination of intraluminal vesicles that are typical for late endosomes depend on the Ca^{2+}-binding proteins annexin 1 and annexin 2. Backfusion of intraluminal vesicles with the limiting membrane of the late endosome also occurs and is used by viruses to escape endosomes and enter the cytosol. ALIX, a potential regulator of backfusion, also requires Ca^{2+}, as does the fusion of late endosomes with lysosomes and the reformation of lysosomes from hybrid organelles. The latter processes might require the endosomal Ca^{2+} channel TRPML1. Lysosomes, unlike endosomes, have a high concentration of Ca^{2+}. Possibly, they can saturate their lumen with Ca^{2+} (filling) that is released from the ER and could therefore act as moderators of ER-derived Ca^{2+} waves (moderation). Lysosomal Ca^{2+} release induces secondary Ca^{2+} release from the ER (initiation). As lysosomes can both take-up Ca^{2+} from the ER and release their own Ca^{2+}, ER–lysosomal Ca^{2+} crosstalk might amplify Ca^{2+} signals from the ER (propagation). The green shape represents the ER.

after lysosomal permeabilization is followed by additional spikes of high cytosolic Ca^{2+} levels. This suggests that the primary Ca^{2+} release from lysosomes triggers a second release from other compartments (Fig. 3). Inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which pumps cytosolic Ca^{2+} into the ER lumen, inhibits secondary oscillations that are induced by Ca^{2+} release from lysosomes, indicating that this second wave involves Ca^{2+} release from the ER (Kilpatrick et al., 2013).

Lysosomes can modulate Ca^{2+} entry into the ER because permeabilization of lysosomes exaggerates the cytosolic Ca^{2+} signals that are evoked by phosphatidylinositol (1,4,5)-triphosphate [PtdIns(1,4,5)P_3], an activator of ER-based PtdIns(1,4,5)P_3 receptors and of Ca^{2+} release from the ER (López-Sanjurjo et al., 2013). Therefore, Ca^{2+} channels in the ER are activated – and intracellular Ca^{2+} waves modulated – by lysosomal Ca^{2+} release and uptake. But can Ca^{2+} release by the ER also induce Ca^{2+} release by lysosomes? Using sea urchin eggs, Morgan and colleagues demonstrated that PtdIns(1,4,5)P_3 and cyclic ADP-ribose (cADPR)-induced Ca^{2+} release from the ER stimulates Ca^{2+} release from acidic organelles (Morgan et al., 2013), indicating that the ER and lysosomes are also coupled in Ca^{2+} signaling. It has been shown that an open Ca^{2+} channel generates a highly localized spike in cytosolic Ca^{2+}, which decays to normal levels within 100 nm from the channel (Shuai and Parker, 2005). This suggests that Ca^{2+} communication between compartments should only occur in a confined space, in this case, probably the ER–lysosome membrane contact sites. Indeed, all of the above studies that addressed the exchange of Ca^{2+} between endosomes and ER, and vice versa, report a close apposition between lysosomes and ER, which suggests that a local exchange of ions occurs in the cleft of MCSs. If endosomes are an additional storage site for Ca^{2+}, Ca^{2+} could also regulate endosomal dynamics as discussed below.

Ca^{2+} in endosomal biology

Buffering of endosomal Ca^{2+} induces lysosomal-storage disorder phenotypes (Lloyd-Evans et al., 2008), indicating that endosomal Ca^{2+} is crucial for endosomal function. However, not much is known about the endosomal processes that require Ca^{2+}. Some reports suggest that Ca^{2+} is required for lysosomal fusion, fusion between late endosomes and lysosomes, and the reformation of lysosomes from hybrid late-endosomal–lysosomal organelles (Pryor et al., 2000) (Fig. 3). Purified late endosomes and lysosomes require Ca^{2+} that is derived from the organelle lumen for fusion, at least in a cell-free system (Pryor et al., 2000). Ca^{2+} might also be important for 'backfusion' of intraluminal vesicles (Fig. 3). Backfusion is a poorly understood process whereby intraluminal vesicles fuse with the limiting membrane of multivesicular late endosomes. In this context, a multivesicular body is a dynamic compartment with intraluminal vesicles that is formed by endosomal sorting complexes required for transport (ESCRT) complexes and reversed (by backfusion) by a presently unknown system. The cytosolic protein ALIX might be required for this backfusion event, and its recruitment to late endosomes requires Ca^{2+} binding (Bissig et al., 2013). ALIX then binds a series of proteins, including ESCRT subunits and lysobisphosphatidic acid (LBPA), a lipid that is selective for late endosomes. Another family of proteins, the annexins, also requires Ca^{2+} for their binding to membranes (Gerke et al., 2005). There are over ten annexins encoded in the human genome and all of them require Ca^{2+} binding for their association to various membranes, implicating Ca^{2+} in many of the membrane-associated processes that annexins are involved in (Gerke et al., 2005). For instance, annexin 2 can bind cholesterol in a Ca^{2+}-dependent manner and regulates multivesicular endosome biogenesis (Mayran et al., 2003), whereas annexin 1 has been implicated in inward vesiculation (Futter et al., 1993). Early endosomal fusion also

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requires Ca^{2+} in a calmodulin- and early endosome antigen-1 (EEA1)-dependent manner (Colombo et al., 1997; Lawe et al., 2003) (Fig. 3).

The endosomal Ca^{2+} channels that allow lysosomal Ca^{2+} uptake and release are unknown, but candidates include the two-pore channels (TPCs), TRPML1 and TRPM2 (Box 2). Interestingly, TPCs and TRPM2 appear to be gated by PtdIns(3,5)P_2, a major determinant of late endosome maturation (Dong et al., 2010; Wang et al., 2012).

These findings collectively suggests that endosomal maturation and Ca^{2+} transfer, analogous to cholesterol transport and endosomal maturation, occurs in a coupled process and also raises the question as to whether ER–endosome contact sites also have a role in late endosomal maturation.

ER in control of endosomal maturation

The relationship between the ER and endosomes appears to be of mutual benefit because cholesterol and Ca^{2+} are shared between the compartments. However, although there are not many known ER functions that are controlled by endosomes [with the exception of the sterol regulatory element-binding protein (SREBP) pathway that are in control of cellular cholesterol synthesis (Brown and Goldstein, 1999)], many steps in endosomal function are controlled by the ER (Fig. 1). A striking example is the control of EGFR signaling. EGFR is activated at the cell surface upon binding of EGF, which induces receptor dimerization, phosphorylation and downstream signaling. EGFR is subsequently internalized, and it maintains its signaling competence until it is dephosphorylated by the ER tyrosine-phosphatase PTP1B and/or sequestered within multivesicular bodies (Eden et al., 2010). In an analogous process, activated G-CSFR is dephosphorylated by PTP1B to downregulate its signaling (Palande et al., 2011). Dephosphorylation of tyrosine-phosphorylated transmembrane receptor proteins by PTP1B at ER–late-endosomes MCSs is probably not limited to EGFR and G-CSFR, as PTP1B has several other known substrates (Yip et al., 2010), including subunits of the ESCRT complex (Hrs and STAM subunits) that are involved in ILV formation (Stuible et al., 2010; Eden et al., 2012). The ER might further control ILV biogenesis through interactions between ORP1L and VAP-A, as ORP1L is also required for ILV formation (Kobuna et al., 2010). Similarly, interactions between ORP5 and Hrs might also regulate ILV formation (Pridgeon et al., 2009). This suggests that ER–endosome contacts control endosomal maturation by terminating receptor signaling and ILV generation for multivesicular body formation.

However, the ER–endosome connections do not stop here; late endosomal transport and fusion steps are also regulated at the ER–late-endosome junction. The cholesterol sensor ORP1L is part of a tri-partite complex that contains the GTPase RAB7 and its effector RILP (Johansson et al., 2005; Johansson et al., 2007). RILP directly binds the dynein motor subunit $p150^{Glued}$ (also known as DCTN1) and thereby recruits the dynein–dynactin motor for minus-end-directed transport of lysosomes (Cantalupo et al., 2001; Jordens et al., 2001; Johansson et al., 2007). VAP-A interacts with ORP1L when the cholesterol levels in late endosomes are low to initiate late-endosome–ER contact sites. At these sites, VAP-A removes $p150^{Glued}$ from RILP, thereby facilitating plus-end-directed transport of late endosomes by kinesin motors (Rocha et al., 2009; Vihervaara et al., 2011). The ER thus coordinates the direction of late endosomal movement, but why is the timing of this process regulated by cholesterol? As cholesterol is released from LDL in the acidic environment of late endosomes, cholesterol might act as maturation signal that induces the transport of mature endosomes towards the minus ends of microtubules [which are typically organized by a microtubule-organizing center (MTOC)]. In non-polarized cells, the MTOC is located adjacent to the nucleus in the center of the cell (Tang and Marshall, 2012). Increases in late endosomal cholesterol allow recruitment of the dynein motor and transport towards the MTOC, and therefore the transport of mature endosomes deeper into cells. This might explain why mature late endosomes and lysosomes are normally localized in the perinuclear region in non-polarized cells (Huotari and Helenius, 2011). RILP also binds the homotypic fusion and vacuole protein sorting (HOPS) complex that is required for late endosomal tethering and fusion (van der Kant et al., 2013b). HOPS recruitment to RILP is also controlled by interactions between ORP1L and VAP-A. As a result, both endosomal transport and endosomal tethering for fusion are controlled in ER–late-endosome contact sites. Why the ER controls late endosomal transport, fusion and ILV formation is unknown. One possibility is that this allows the ER to coordinate endosomal trafficking at the cellular level to ensure coordinated exchange of cholesterol and Ca^{2+} from multiple late endosomes at their correct maturation stage. ER–endosome contacts might also function in autophagy. The ER-resident motor membrane protein complex subunit 6 (ECM6) regulates autophagosome formation and interacts with the early endosomal regulator RAB5 (Li et al., 2013). RAB5 and EMC6 have been found to colocalize on punctuated structures that are also labeled with ER markers, but high-resolution microscopy analysis (Fig. 4) will be required to understand whether these punctae reflect RAB5 recruitment to the ER, or ER–endosome contact sites.

As ER–endosome contact sites are involved in many steps in endosomal biogenesis, mutations in proteins that are thought to

Box 2. Tuning in on the right channel: what is the lysosomal Ca^{2+} channel?

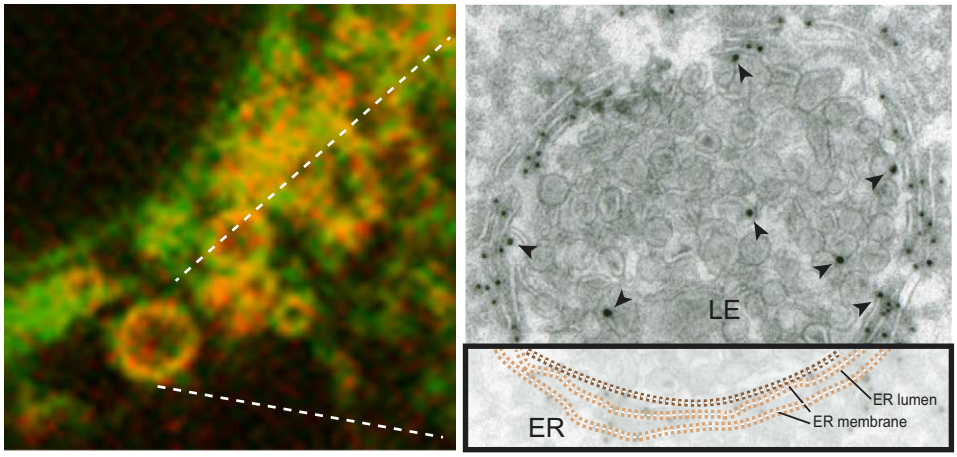
Nicotinic acid adenine dinucleotide phosphate (NAADP) is an intracellular messenger that can induce the release of Ca^{2+} from intracellular structures. Although the potential of NAADP to release intracellular Ca^{2+} has been known since the late eighties (Clapper et al., 1987), its cellular target has remained unknown for a long time. In 2002, NAADP was found to mobilize Ca^{2+} from lysosome-related organelles in sea urchin eggs (Churchill et al., 2002), and later reports have verified NAADP-induced lysosomal Ca^{2+} release in other cells (Patel and Docampo, 2010). TPC proteins were originally identified as NAADP receptors, but later studies showed that TPCs are not activated by NAADP, but are PtdIns(3,5)P_2-activated Na^+-selective ion channels (Wang et al., 2012). The mucolipin transient receptor potential (TRPML) channel is another endo/lysosome-localized Ca^{2+} pump that might contribute to endosomal Ca^{2+} release. Mutations in TRPML1 cause mucolipidosis type IV (ML4), a severe neurodegenerative disease (Sun et al., 2000). TRPML1 activation is also regulated by PtdIns(3,5)P_2 levels. TRPM2 functions as a lysosomal Ca^{2+}-release channel in beta cells (Lange et al., 2009). For additional reading see Patel and Brailoiu (Patel and Brailoiu, 2012). Whether and how NAADP is involved in the opening of lysosomal Ca^{2+} channels and whether additional lysosomal Ca^{2+} channels exist is an open question at present.

function at ER–endosome contacts result in severe diseases as discussed below.

Endosomes and the ER – what happens if the connection is lost?

Niemann-Pick type C disease, caused by mutations in the genes *NPC1* or *NPC2*, is one disease in which late endosome–ER crosstalk is likely disrupted. As discussed above, NPC1 interacts directly with the ER protein ORP5. This suggests that the absence of NPC1, as in Niemann–Pick type C disease, could directly alter contact sites. Although this concept has not been tested to our knowledge, it has been shown that absence or functional impairment of NPC1 results in the accumulation of cholesterol in the late endosomal membrane, and, hence, impairs contact site formation between ORP1L and VAP-A (Rocha et al., 2009). This has several effects downstream, such as an increased minus-end transport of late endosomes (Mukherjee and Maxfield, 2004; Rocha et al., 2009) and possibly impairments in endosomal fusion (Huynh et al., 2008), as mediated by the HOPS complex (van der Kant et al., 2013b). A reduction in the fusiogenic capacity of lysosomes might explain the observed impairment of autophagosome clearance in cells of Niemann Pick type C disease patients (Ordonez et al., 2012), a process that requires lysosome–autophagosome fusion. The impaired function of ORP1L in NPC1-deficient cells might also result in the disruption of ILV formation, thus increasing the size of late endosomes, which is observed in these cells (Ko et al., 2001). Another consequence of the loss of ER–late-endosome contact sites might be an impaired Ca^{2+} transfer between the ER and late endosomes. Indeed, NPC1-deficient cells have lower levels of lysosomal Ca^{2+} (Lloyd-Evans et al., 2008), and any disturbances in Ca^{2+} levels precede measurable changes in cholesterol levels in cells that have been treated with U18666A (a compound known to induce a Niemann–Pick type C disease-like phenotype in cells). However, it is worth noting that the mode of action of U18666A is not completely understood and it might have downstream effects that are not based on abrogating NPC1 function. Nevertheless, these studies indicate that lysosomal Ca^{2+} signaling and cholesterol metabolism are intimately linked with either the absence of Ca^{2+}, which induces the accumulation of cholesterol, or an accumulation of cholesterol that causes a depletion of Ca^{2+}. For instance, mutations in the Ca^{2+} transporter TRPML1 protein (which is mutated in mucolipidosis type IV) have been shown to affect endosomal lipid handling, endosomal maturation and endosomal transport (Bargal and Bach, 1997; Shen et al., 2012).

Endosomal disruptions are also among the first clinical observations in Alzheimer's disease (Cataldo et al., 2000; Israel et al., 2012). Recent data indicate that presenilin 1, a protein mutated in familiar Alzheimer's disease (FAD), is involved in the regulation of lysosomal Ca^{2+} homeostasis (Tu et al., 2006; Coen et al., 2012). Presenilin 1 is a transmembrane protein that is located in several intracellular organelles including the ER (Annaert et al., 1999). One possibility is that presenilin 1 has a role in the communication between the ER and late endosomes to modulate lysosomal function. Late endosomal proteins frequently exhibit contacts with VAP-A that result in the initiation of contact sites. VAP-A also has a close, but poorly studied homologue, VAP-B, which has been implicated in disease. For example, mutations in VAP-B cause amyotrophic lateral sclerosis (ALS) type 8, a severe neurodegenerative disorder that affects long motor neurons. Mutant VAP-B sequesters wild-type VAP-A into aggregates (Teuling et al., 2007), possibly preventing VAP-A from initiating contact sites between the ER and late endosomes or other organelles. Neuronal ceroid lipofuscinoses (NCLs) are another group of neurodegenerative disorders. Here, lipopigments – a mix of protein and lipids – accumulate in late endosomes and patients suffer from progressive loss of motor and psychological ability. Although most neuronal ceroid lipofuscinoses (CLN)



Confocal micropy
Green: HA–VAP-A (ER protein)
Red: GFP–ORP1L (late endosomal protein)

Immunoelectron microscopy
Small gold particles: HA–VAP-A (ER protein)
Large gold particles (arrowheads):
GFP–ORP1 (late endosomal protein)

Fig. 4. Contact site or fusion? Many organelles make transient – non-continuous – contacts with the ER. These can be easily observed by confocal microscopy as shown in the left image. Here, the late endosome compartment is labeled differently from the ER by expression of a HA-tagged version of the late endosomal ORP1L (labeled in green) and a GFP variant of the ER protein VAP-A (labeled in red). ORP1L and VAP-A perfectly colocalize suggesting that they are on the same membrane. Because the resolution of light microscopy is usually >240 nm, details below this scale remain unresolved. However, when the same cells are analyzed at higher resolution by immunoelectronmicroscopy (shown on the right), the formation of membrane contact sites (rather than fusion) between late endosomes and ER is apparent because HA–ORP1L (large gold particles, indicated with arrowheads) and GFP–VAP-A (small gold particles) label exclusively the endosomes and ER, respectively.

proteins have been localized to endosomal compartments and function either as pumps, enzymes or modulators of endosomal transport (Getty and Pearce, 2011; Uusi-Rauva et al., 2012); two CLN proteins, CLN6 and CLN8, are actually ER proteins (Getty and Pearce, 2011). Without an understanding of ER–endosomal contact sites, it would be difficult to appreciate how ER proteins can induce a lysosomal storage disorder. The recent insights into ER–late-endosome contact sites and their possible functions provide an explanation for how these ER proteins could contribute to endosomal homeostasis. Further studies will reveal whether and how mutations in CLN6 and CLN8 disrupt the crosstalk between endosomes and the ER. As CLN8 has been reported to interact with VAP-A (Passantino et al., 2013), a direct contact between ER and endosomes is a plausible mechanism. Interestingly, all the above diseases affect mainly the brain, indicating either that neurons are more vulnerable to disruptions in ER–endosome MCSs or that there are disruptions in endosomal transport that are regulated by these contacts (Neefjes and van der Kant, 2014).

Concluding remarks

Although intracellular compartments were originally considered separate and independent structures, recent insights have revealed that there is intercompartmental control of many cellular processes. The ER, as the most abundant compartment, appears to be a master controller of different compartments. The ER might act as a 'middleman' by allowing the lateral transfer of hydrophobic structures from endosomes to other ER-connected compartments, such as mitochondria, Golgi and the plasma membrane. Virtually all late endosomes are dynamically connected to the ER, and the ER might be involved in additional endosomal processes, such as exchange of information regarding nutrient state, infection, transport and other processes via MCSs. At present we do not understand if – and how – diverse functions of ER–endosome interactions such as Ca^{2+} exchange, cholesterol transfer and endosomal maturation are coordinated at the ER–endosome interface. For example, how does data integration occur?

ER–endosome contact sites not only allow the ER to be informed of the extracellular world (by message relay through signaling- and cargo-containing endosomes), but also to employ the endosomal system to retrieve essential components that are continuously released from the ER through the secretory pathway. Although cholesterol is the best-studied small molecule that is transferred between compartments, it is likely that other nutrients and building blocks are also transferred between ER and endosomes. What exactly is transferred between the two membranes in ER–late-endosomal contact sites beyond Ca^{2+} and cholesterol is at present unclear.

The regulation of interactions between endosomes and the ER is a relatively novel concept that has major implications for our understanding of the dynamic state of cellular compartments. We believe that the intracellular system should be considered as a network of many interacting compartments. Individual endosomes are not only 'connected' to other endosomes, via the ER, but are also connected with other organelles such as mitochondria, plasma membrane and Golgi that also contact the ER. This view might help to explain complex diseases, in which multiple organelles ranging from the ER to endosomes and mitochondria are affected.

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