Review

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Sealing holes in cellular membranes

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Abstract

The compartmentalization of eukaryotic cells, which is essential for their viability and functions, is ensured by single or double bilayer membranes that separate the cell from the exterior and form boundaries between the cell's organelles and the cytosol. Nascent nuclear envelopes and autophagosomes, which both are enveloped by double membranes, need to be sealed during the late stage of their biogenesis. On the other hand, the integrity of cellular membranes such as the plasma membrane, lysosomes and the nuclear envelope can be compromised by pathogens, chemicals, radiation, inflammatory responses and mechanical stress. There are cellular programmes that restore membrane integrity after injury. Here, we review cellular mechanisms that have evolved to maintain membrane integrity during organelle biogenesis and after injury, including membrane scission mediated by the endosomal sorting complex required for transport (ESCRT), vesicle patching and endocytosis.

Keywords autophagy; endocytosis; ESCRT; lysosome; membrane repair
Subject Category Membranes & Trafficking
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Introduction

The bilayered membranes of eukaryotic cells are vital to their very existence, with the plasma membrane separating the cells from their surroundings, and the endomembranes enclosing the various organelles. It is crucial that these membranes are intact so that only gated transport of molecules and ions across them can occur. Defective membrane sealing is indeed associated with a large number of diseases, including myopathies, central and peripheral neurological disorders, and coronary diseases (Cooper & McNeil, 2015). Sealing of holes in membranes is therefore of great importance in biology, both during biogenesis of double-membrane organelles and as response to membrane damage. For instance, all newly formed nuclei contain transient holes (Anderson & Hetzer, 2008), and it has been estimated that as many as 20–30% of cardiac and skeletal muscle cells and 6% of skin cells have transient openings in their plasma membrane (McNeil & Steinhardt, 1997). Whereas membrane openings during organelle biogenesis have a defined size of < 100 nm (Olmos *et al,* 2015; Denais *et al,* 2016), holes in membranes due to damage may range from a few nm to several μm (Cooper & McNeil, 2015). The molecular and cellular mechanisms that have evolved to seal such holes will be discussed in this review.

Mechanisms of membrane sealing

Holes in bilayer membranes can be sealed by several alternative mechanisms (Fig 1), most of which require specialized protein machineries. Below is a brief overview of different mechanisms that have been proposed.

Self-sealing of membranes

Studies of artificial membranes have shown that ruptured bilayer membranes can seal spontaneously without the need for auxiliary proteins (Gozen & Dommersnes, 2014) (Fig 1A). When a hole is formed in a lipid bilayer, lipid disorder present on the curved edges of the hole provides a driving force for resealing so that edge tension can be minimized. On the other hand, resealing is counteracted by membrane tension, which is low in most artificial membranes but is high in a cellular context where the membrane is attached to rigid components such as the cytoskeleton or the nuclear lamina. The high tension of cellular membranes thus disfavours self-sealing of holes of more than a few nm (McNeil & Kirchhausen, 2005).

Reduction in membrane tension

Because membrane tension limits the ability of cellular membranes to self-seal, cellular mechanisms that reduce membrane tension can promote membrane sealing. One such mechanism is addition of more membrane via vesicle fusion (Fig 1B). A second mechanism for reducing membrane tension is to alter membrane curvature, whereas a third mechanism is to disrupt membrane-associated protein scaffolds.

Patching by fusion of intracellular membranes

Early work on membrane repair revealed numerous small cytoplasmic vesicles close to the site of injury, that formed larger vesicles via homotypic fusion (Terasaki *et al,* 1997) (Fig 1C). This generated the hypothesis that large cytoplasmic vesicles can patch up

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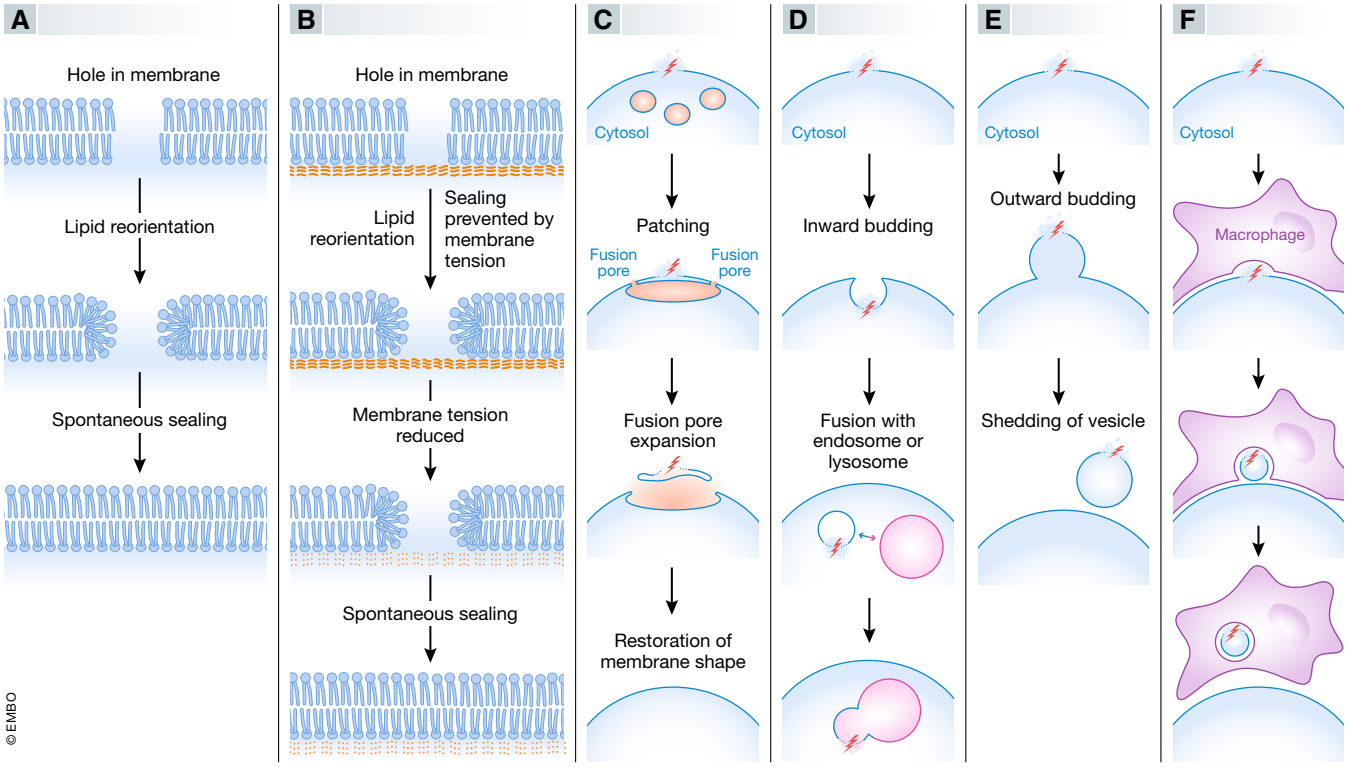

Figure 1. Mechanisms of membrane sealing.

Holes in lipid bilayers can be sealed by several alternative mechanisms, including membrane self-sealing (A), reduction in membrane tension to promote self-sealing (B), patching of hole by vertex fusion of large intracellular vesicles (C), inward budding of hole-containing membrane area (D), outward budding of hole-containing membrane area (E), or removal of hole-containing membrane area by adjacent cell (F).

large (up to several μm) damaged membrane areas. It has been proposed that the patch vesicle makes fusion pores with the plasma membrane at several discrete sites around the damaged area and that these pores expand to form a continuous union of patch vesicle and the plasma membrane. In this way, the damaged plasma membrane will be replaced by the new membrane (McNeil & Kirchhausen, 2005). This mechanism is still debated as it would require stabilization and lateral expansion of the fusion pore to form a ring around the site of damage that is shed when the fusion pores meets. An alternative mechanism for how vesicle fusion might mediate membrane repair is the concept that such fusion causes release of an enzyme that promotes membrane sealing (Tam *et al,* 2010).

Inward budding and scission of damaged membrane area

One of the proposed mechanisms for membrane repair entails inward budding (i.e., in the direction towards the cytosol) of a membrane area that contains the damaged zone (Idone *et al,* 2008) (Fig 1D). The resulting cytoplasmic vesicle, which will contain a damaged membrane area, can then fuse with an endosome or lysosome, thus effectively transferring the damage from the original membrane to the endosome or lysosome membrane, from where it can be internalized into the lumen in the form of an intraluminal vesicle (ILV) (Andrews *et al,* 2014).

Outward budding and scission of damaged membrane area

Outward vesicle budding (i.e. in the direction away from the cytosol) ensures that damaged membrane areas are removed from their site of origin and shed either as extracellular vesicles or ILVs that form inside organelles such as endosomes or lysosomes (Fig 1E). This type of vesicle budding shares topology with closure of nascent double-membrane organelles such as autophagosomes and the nuclear envelope and is mediated by the same molecular machinery.

Removal of damaged membrane area by adjacent cells

Even though membrane repair has mostly been described as a cell autonomous mechanism, muscle cells, which are particularly prone to damage of their plasma membrane, can also engage neighbouring macrophages to pinch off damaged portions of the muscle cell plasma membrane (Middel *et al,* 2016) (Fig 1F). How macrophage-mediated removal of the damaged plasma membrane can proceed in a way that preserves membrane integrity still remains to be resolved.

Proteins that promote membrane sealing

Proteins that promote membrane sealing can roughly be subdivided into detectors of membrane damage, fusion regulators and fission regulators (see Table 1).

Table 1. Proteins involved in membrane sealing. See text for references.

General function in membrane sealing		Protein name	Specific function	Site of action
Sensing of membrane integrity	Sensing of Ca^{2+} influx into cytosol	PDCD6	Binds to ALIX to promote ESCRT recruitment	Plasma membrane, endolysosomes
		ALIX	Promotes ESCRT recruitment	Plasma membrane, endolysosomes
		Annexins	Together with Ca^{2+} binding proteins of the S100 family, involved in membrane fusion, shaping, sealing and tension reduction	Plasma membrane, endolysosomes
		SYT7	Endolysosomal protein that activates SNAREs in membrane fusion	Plasma membrane
		Dysferlin	Accumulates phosphatidylserine at the site of membrane damage, as an "eat-me" signal for macrophages. Interacts with some annexins	Plasma membrane
		TMEM16F	Lipid scramblase that causes exposure of phosphatidylserine at the extracytosolic leaflet	Plasma membrane
	Sensing of β-galactoside sugars on membrane proteins not normally exposed to cytosol	GAL3	Interacts with ALIX and the autophagy-associated ubiquitin E3 ligase TRIM16	Endolysosomes
		GAL8	Inhibits mTORC1 and interacts with the autophagy receptor NDP52	Endolysosomes
		GAL9	Activates AMPK	Endolysosomes of macrophages and hematopoietic cells
	Sensing of nuclear envelope integrity	CHMP7	Binds to the inner nuclear membrane proteins LEMD2 and LEMD3 and recruits ESCRT-III proteins	Nuclear envelope, micronuclear envelope
Membrane fusion		SNARE proteins (VAMP, syntaxin and SNAP families)	Mediate fusion of specific membranes through formation of tetrahelical trans-SNARE complexes	Multiple
Membrane scission towards cytosol		Acidic sphingomyelinase	Lysosomal enzyme that is shed by Ca^{2+}-dependent exocytosis in response to wounding. Converts sphingomyelin into ceramide, which triggers caveolae endocytosis	Plasma membrane
		GRAF1	GTPase-activating protein for the small GTPase Cdc42. Promotes clathrin-independent endocytosis	Plasma membrane, endosomes
Membrane scission away from cytosol		ESCRT-I	Recruits ESCRT-III	Multiple
		ESCRT-III	Filamentous complex that mediates scission of narrow membrane necks filled with cytosol, to achieve membrane sealing	Multiple
		VPS4	AAA ATPase that regulates ESCRT-III assembly and disassembly	Multiple

Ca^{2+}-binding proteins as sensors of membrane damage

Whereas cytosolic levels of Ca^{2+} are low (about 100 nM), the Ca^{2+} concentrations in extracellular fluids and organelles such as the endoplasmic reticulum (ER) and lysosomes can reach several mM (Kass & Orrenius, 1999). The steep Ca^{2+} gradients across cellular membranes indeed form the basis of a simple yet powerful mechanism for detection of membrane integrity. Leakage of Ca^{2+} into cytosol is detected by several Ca^{2+}–binding proteins that promote membrane sealing by membrane fusion, fission or tension reduction.

Annexins are abundant Ca^{2+}-binding cytosolic proteins that tend to assemble on membranes in response to rises in cytosolic Ca^{2+} levels (Gerke & Moss, 2002). Several annexins bind to small Ca^{2+}-binding proteins of the S100 family, which contribute to their functions as Ca^{2+} effectors. Evidence from both lower organisms such as *Paramecium* and mammalian cells has shown that annexins play roles in membrane fusion, shaping and sealing although it has been difficult to pinpoint their exact functional mechanisms (Koerdt *et al,* 2019; Bendix *et al,* 2020). One of the functions of annexins in membrane sealing may be to reduce membrane tension so that membrane self-sealing is promoted (Bouter *et al,* 2011). Annexins have also been proposed to assemble into multimeric structures that physically cap the hole in the membrane (Demonbreun *et al,* 2016; Robinson *et al,* 2020).

Synaptotagmins are integral membrane proteins that function as Ca^{2+}-sensors that mediate membrane fusion. Among these, Synaptotagmin-7 (SYT7) has been found to be particularly important for membrane repair. The cytosolic part of SYT7 contains two Ca^{2+}- and

phospholipid-binding C2 domains that sense cytosolic $Ca^{2+}$and mediate interactions with membranes. Like other members of the Synaptotagmin family, SYT7 regulates formation of SNARE complexes that drive membrane fusion and thus transduces Ca^{2+} sensing into membrane fusion (see below).

Whereas SYT7 couples Ca^{2+} sensing to membrane fusion in membrane repair, another Ca^{2+} sensor, PDCD6 (also known as ALG-2), connects with a membrane fission machinery that mediates repair. PDCD6 is a small penta-EF-hand protein that contains a binding site for Ca^{2+} and Mg^{2+} and interacts with other proteins in a Ca^{2+}-dependent manner. One of the interacting partners of PDCD6 is ALIX (PDCD6IP), a scaffolding protein involved in diverse cellular functions. ALIX also binds Ca^{2+}, albeit with lower affinity than PDCD6. Injury-triggered influx of Ca^{2+} causes accumulation of PDCD6 at the site of injury, and PDCD6 in turn recruits ALIX. ALIX then recruits components of the ESCRT machinery that mediates membrane repair by outward budding and fission of the damaged membrane area (see below).

Muscle cells contain an additional Ca^{2+}-sensing membrane protein, Dysferlin (DYSF). The cytosolic region of DYSF harbours seven C2 domains, and Ca^{2+} sensing by DYSF plays a key role in repair of damaged plasma membranes of muscle cells as illustrated by the involvement of DYSF mutations in genetic myopathies (Cardenas *et al,* 2016). DYSF has both cell autonomous and non-autonomous functions in membrane sealing. Via an arginine-rich motif, DYSF mediates accumulation of phosphatidylserine at the site of plasma membrane damage, and this triggers removal of the damaged membrane region by neighbouring macrophages (Middel *et al,* 2016). Translocation of phosphatidylserine to the extracytoplasmic leaflet can be mediated by a Ca^{2+} –activated lipid scramblase, TMEM16F, which promotes repair of the plasma membrane after injury by pore-forming toxins by reducing membrane tension and facilitating release of extracellular vesicles containing damaged membranes (Wu *et al,* 2020).

Galectins as sensors of endomembrane damage

Galectins are a family of cytosolic lectins that bind specifically to β-galactoside carbohydrates, which are linked to the extracytosolic domains of a large number of integral membrane proteins (Vasta, 2009). They function as pattern recognition receptors and can serve as sensors of membrane integrity by binding to sugars that are not normally exposed to cytosol. This has been found to be the case with the ubiquitously expressed Galectin-3 and Galectin-8, and also with Galectin-9, which is mainly expressed in macrophages and cells of the haematopoietic system. Galectins can transmit signals about compromised membrane integrity in several different ways. For example, upon lysosome damage, Galectin-3 interacts with ALIX and the autophagy-associated E3 ubiquitin ligase TRIM16 (Chauhan *et al,* 2016; Jia *et al,* 2020b), Galectin-8 inhibits the mTORC1 signalling complex and interacts with the autophagy receptor NDP52 (Jia *et al,* 2018; Weng *et al,* 2018), and Galectin-9 activates the low energy-sensing kinase AMPK (Jia *et al,* 2020a). By these mechanisms, galectins can couple sensing of membrane damage to membrane repair but also to metabolic control and autophagy of damaged organelles.

SNARE proteins in membrane fusion during membrane sealing

Soluble NSF attachment protein receptors (SNAREs) are a large group of small proteins that mediate membrane fusion (Sudhof & Rothman, 2009). They are frequently subdivided into vesicle (v)-SNAREs (synaptobrevin/VAMP family) and target membrane (t)-SNAREs (Syntaxin and SNAP families). Most SNAREs are integral membrane proteins that are tail-anchored to specific membranes, but there are also examples of soluble SNAREs and SNAREs that are membrane-bound via cysteine-linked palmitoyl chains. All SNAREs are characterized by their content of a 60–70 amino acid region called the SNARE motif, which contains heptad repeats. The well-characterized core trans-SNARE complex in fusion of synaptic vesicles with the presynaptic plasma membrane consists of a four-α-helix bundle with one α-helix contributed by the synaptic vesicle protein VAMP2, one helix by the plasma membrane protein STX1A, and two α-helices by the plasma membrane protein SNAP-25 (Poirier *et al,* 1998). SNARE assembly into stable four-helix bundles not only bridges membranes but also provides the energy for initiating membrane fusion.

ESCRT proteins in membrane scission and sealing

Originally identified for its function in endosomal sorting of ubiquitinated membrane proteins into ILVs of endosomes (Katzmann *et al,* 2002), the endosomal sorting complex required for transport (ESCRT) machinery is now recognized as the major catalyst of scission and sealing reactions at diverse cellular locations that share the same topology (Schoneberg *et al,* 2017; Vietri *et al,* 2020a). The machinery consists of three subcomplexes, ESCRT-I, -II and –III. ESCRT-I is an elongated helical heterotetramer with the ubiquitin-binding protein TSG101 as a core component. ESCRT-II is a heterotetramer of three winged-helix (WH) domain proteins (EAP30, EAP45 and two subunits of EAP20), whereas ESCRT-III is a biochemically more heterogenous complex that forms helical filaments consisting of small highly charged α-helical proteins of the CHMP family. The membrane scission/sealing activity of ESCRT is mediated by the ESCRT-III filaments, whose activity is controlled and modulated by the AAA ATPase VPS4. ESCRT-III can be recruited to membranes by several alternative mechanisms, including direct interactions with ESCRT-I, ESCRT-II, the ESCRT-II/-III like protein CHMP7 or the ESCRT-I-binding protein ALIX.

During sealing of damaged plasma membrane and lysosome membranes, both the ESCRT-I subunit TSG101 and ALIX have been assigned important functions in ESCRT-III recruitment (Jimenez *et al,* 2014; Scheffer *et al,* 2014; Radulovic *et al,* 2018; Skowyra *et al,* 2018), whereas the Ca^{2+} sensor PDCD6 and the carbohydrate sensor GAL3 have been implicated in ALIX recruitment (Scheffer *et al,* 2014; Skowyra *et al,* 2018; Jia *et al,* 2020b). ESCRT-I also plays a pivotal role in ESCRT-III recruitment for closure of nascent autophagosomes (Takahashi *et al,* 2019). During reformation or repair of nuclear envelopes, CHMP7, a protein with both ESCRT-II- and ESCRT-III-like domains, functions as a sensor for openings in the nuclear envelope and mediates recruitment of ESCRT-III (Vietri *et al,* 2015; Denais *et al,* 2016; Olmos *et al,* 2016; Raab *et al,* 2016; Vietri *et al,* 2020b). Available data suggest that the ESCRT machinery is suited for sealing small holes in membranes, with a diameter < 100 nm (Jimenez *et al,* 2014; Olmos *et al,* 2015; Denais *et al,* 2016; Bohannon & Hanson, 2020), whereas other mechanisms probably account for sealing of larger holes. It is interesting to note that low membrane tension promotes ESCRT-III recruitment, and reduction in membrane tension after membrane injury could thus by itself contribute to ESCRT-III recruitment (Mercier *et al,* 2020).

Membrane sealing during organelle biogenesis

Most of the cell's organelles form by membrane budding and fusion reactions that do not involve membrane sealing. However, two organelles enclosed by double membranes, the nucleus and the autophagosome, contain holes that need to be closed during the end of their biogenesis.

Sealing of the nascent nuclear envelope

During mitosis in mammalian cells, duplicated chromosomes condense during prophase, and the nuclear envelope is disassembled. The chromosomes align at the equator of the dividing cell in metaphase and are then pulled by microtubule bundles to each pole in anaphase. At the end of anaphase, new nuclear envelopes assemble around the two separated chromosome clusters to form daughter nuclei, which are completed during telophase. This is followed by separation of the two daughter cells at the end of cytokinesis. The nascent nuclear envelopes are formed from membranes mobilized from the endoplasmic reticulum, which fuse to form a continuum. However, the completion of the new nuclear envelopes requires both the removal of the microtubule bundles used for chromosome separation and the closure of holes remaining after the microtubules have been removed (De Magistris & Antonin, 2018). Both these events are accomplished by the ESCRT machinery (Fig 2A).

Firstly, ESCRT-III is recruited and its polymerization triggered to the microtubule-traversed holes. This requires the interaction of endoplasmic reticulum-associated CHMP7 with the integral inner nuclear membrane protein LEMD2 (Vietri *et al,* 2015; Olmos *et al,* 2016; Webster *et al,* 2016; Gu *et al,* 2017). ESCRT-III subsequently recruits the microtubule-severing AAA ATPase Spastin via the ESCRT-III associated protein IST1, and this causes removal of the spindle microtubules (Vietri *et al,* 2015). The remaining holes are then closed by ESCRT-III (Olmos *et al,* 2015; Vietri *et al,* 2015).

Regulation of these events in time and space requires the interaction of CHMP7 with LEMD2. LEMD2 bridges the nuclear envelope with the underlying chromatin through an N-terminal Lap2, Emerin, Man1 (LEM)-domain. LEMD2 also contains a C-terminal WH domain, responsible for interaction and activation of CHMP7. At the sites where the membrane is intersected by microtubule bundles, LEMD2 accumulates and undergoes liquid-phase separation, thereby triggering CHMP7 activation and ESCRT-III assembly (von Appen *et al,* 2020). Furthermore, ESCRT-III recruitment and activity are regulated by additional factors, including CC2D1B, which prevents premature ESCRT-III polymerization (Ventimiglia *et al,* 2018), and UFD1, a co-factor of the AAA ATPase p97 that has been shown to regulate ESCRT-III subunit CHMP2A into ESCRT-III filaments (Olmos *et al,* 2015).

Because the nuclear envelope is a double membrane, this ESCRT-mediated closure is topologically equivalent to ESCRT-III-dependent fission reactions such as shedding of extracellular vesicles from the plasma membrane or formation of intraluminal vesicles in endosomes (Vietri *et al,* 2020a). ESCRT-III driven nuclear envelope sealing after mitosis is evolutionarily conserved and has even been described in lower eukaryotes. In semi-open mitosis of *Schizosaccharomyces japonicus,* similar events leading to re-establishment of nucleocytoplasmic compartmentalization in mitotic exit have been described, including the accumulation of a LEMD2 orthologue at intersections between the nuclear envelope and the mitotic spindle, as well as the importance of orthologues of CHMP7, CHMP4B and VPS4 (Yam *et al,* 2011; Pieper *et al,* 2020).

Sealing of the nascent autophagosome

Autophagy is a vital cellular process for lysosomal degradation of cytoplasmic content, which ensures cell survival under starvation conditions and removal of potentially harmful cytoplasmic objects such as microorganisms, protein aggregates and damaged organelles (Mizushima *et al,* 2008; Mizushima *et al,* 2011). The process starts with a double-membrane phagophore membrane that forms around bulk cytoplasm or specific cargo, and the phagophore eventually closes to form a complete autophagosome. When the autophagosome fuses with a lysosome to form an autolysosome, the sequestered cargo becomes degraded by lysosomal hydrolases. Even though contributions to the phagophore membrane may come from the plasma membrane, endosomes, mitochondria or de novo synthesis, most studies to date indicate the endoplasmic reticulum as a major membrane source (Melia *et al,* 2020). When the phagophore membrane has grown around cytoplasmic content and shaped to form a complete autophagosome, it contains a small hole that needs to be closed (Knorr *et al,* 2015). This is achieved by ESCRT-III by a mechanism that is topologically similar to other ESCRT-dependent fission reactions (Takahashi *et al,* 2018; Zhen *et al,* 2020) (Fig 2B). Recruitment of ESCRT-III to the nascent autophagosome requires ESCRT-I, and a putative ubiquitin E2 variant (UEV) domain of the ESCRT-I subunit VPS37A is important for autophagosome closure (Takahashi *et al,* 2019). How ESCRT-I is recruited to the phagophore still remains to be understood, but studies of budding yeast mutants have suggested the involvement of the small endosomal GTPase Rab5 and Atg17, a subunit of the Atg1 autophagic kinase complex, as upstream regulators (Zhou *et al,* 2017; Zhou *et al,* 2019). Because autophagosomes are known to fuse with endosomes (Berg *et al,* 1998), it still remains to be established whether Rab5 and Atg17 (or its mammalian homologue, FIP200) are directly involved in ESCRT recruitment to nascent autophagosomes. The importance of ESCRT-mediated autophagosome sealing is illustrated by the fact that ESCRT depletion causes accumulation of autophagosomes that are incapable of fusing with lysosomes (Filimonenko *et al,* 2007; Rusten *et al,* 2007). This phenomenon can probably be explained by the failure of unsealed autophagosomes to recruit Syntaxin 17, a SNARE protein required for autophagosome-lysosome fusion (Itakura *et al,* 2012).

Membrane repair

Most cellular membranes are exposed to damage, and repair machineries promote cell survival by closing the holes. Below, we will discuss those membranes that have been most studied in the context of damage and repair.

Repair of the damaged plasma membrane

Because the plasma membrane faces the exterior of the cell, it is particularly prone to rupture by pathogens, chemicals and mechanical forces. In addition, holes in the plasma membrane are also employed in inflammatory cell death pathways, including complement-induced necrosis, inflammasome-induced pyroptosis and tumour necrosis factor α-induced necroptosis (Liu & Lieberman,

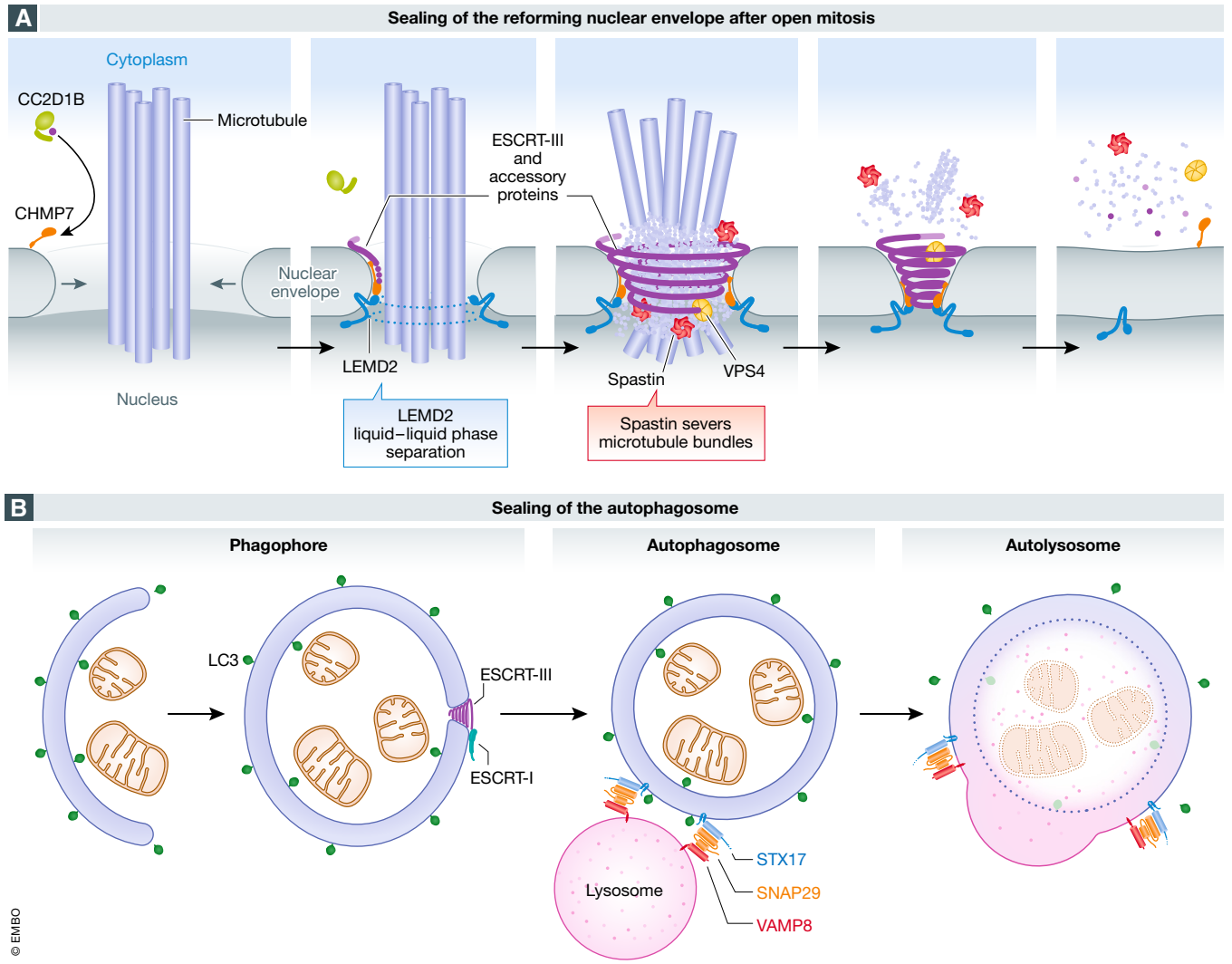

Figure 2. Sealing of holes during biogenesis of double-membrane organelles.

(A) sealing of the reforming nuclear envelope after open mitosis. During anaphase, when the reforming nuclear envelope meets microtubule bundles still connected to chromatin, the inner nuclear membrane protein LEMD2 undergoes liquid-phase separation and activates the ESCRT-III specific nuclear envelope recruitment factor CHMP7, which drives ESCRT-III polymerization. Timing of ESCRT-III recruitment is also regulated by CC2D1B, which prevents its premature localization to the reforming membrane. Spastin recruitment by ESCRT-III is required for severing of mitotic spindle microtubules, while VPS4 modelling of ESCRT-III filaments promotes membrane constriction and sealing. (B) sealing of the autophagosome. During autophagy, the double-membrane phagophore expands to sequester cytoplasmic material for degradation. When the resulting LC3-positive autophagosome is complete, a small hole remains. This hole is sealed by ESCRT-III, which is recruited by ESCRT-I. Sealing is followed by recruitment to the autophagosome membrane of the SNARE protein STX17, which forms a complex with the cytosolic SNARE SNAP29 and the lysosomal SNARE VAMP8, and this mediates fusion of the autophagosome with the lysosome to form an autolysosome.

2020). Several mechanisms have been described for repair of damaged plasma membranes, including patching by intracellular membranes or removal of damaged area by endocytosis, outward budding or pinching-off by neighbouring macrophages (Fig 3).

The involvement of vesicle fusion in plasma membrane repair originally led to the hypothesis that large intracellular vesicles, formed by homotypic fusion of smaller vesicles, patch up the damaged area of the plasma membrane (Terasaki *et al,* 1997; McNeil *et al,* 2000). Indeed, exocytosis of lysosomes plays a well-documented role in plasma membrane repair after mechanical or bacterial toxin-induced injury. Ca^{2+} influx through holes in the plasma membrane activates SYT7 to promote fusion between peripheral lysosomes and the plasma membrane, mediated by a trans-SNARE complex consisting of the lysomal v-SNARE VAMP7 and the plasma membrane t-SNAREs STX4 and SNAP23 (Reddy *et al,* 2001; Rao *et al,* 2004). Interference with SYT7 or SNARE functions prevents plasma membrane repair, demonstrating that fusion between lysosomes and the plasma membrane is essential. It has been difficult to explain how addition of more membrane to the plasma membrane would seal large holes, and also how this would serve to close long-lasting pores formed by bacterial toxins. However, a mechanism that involves formation of expanding fusion

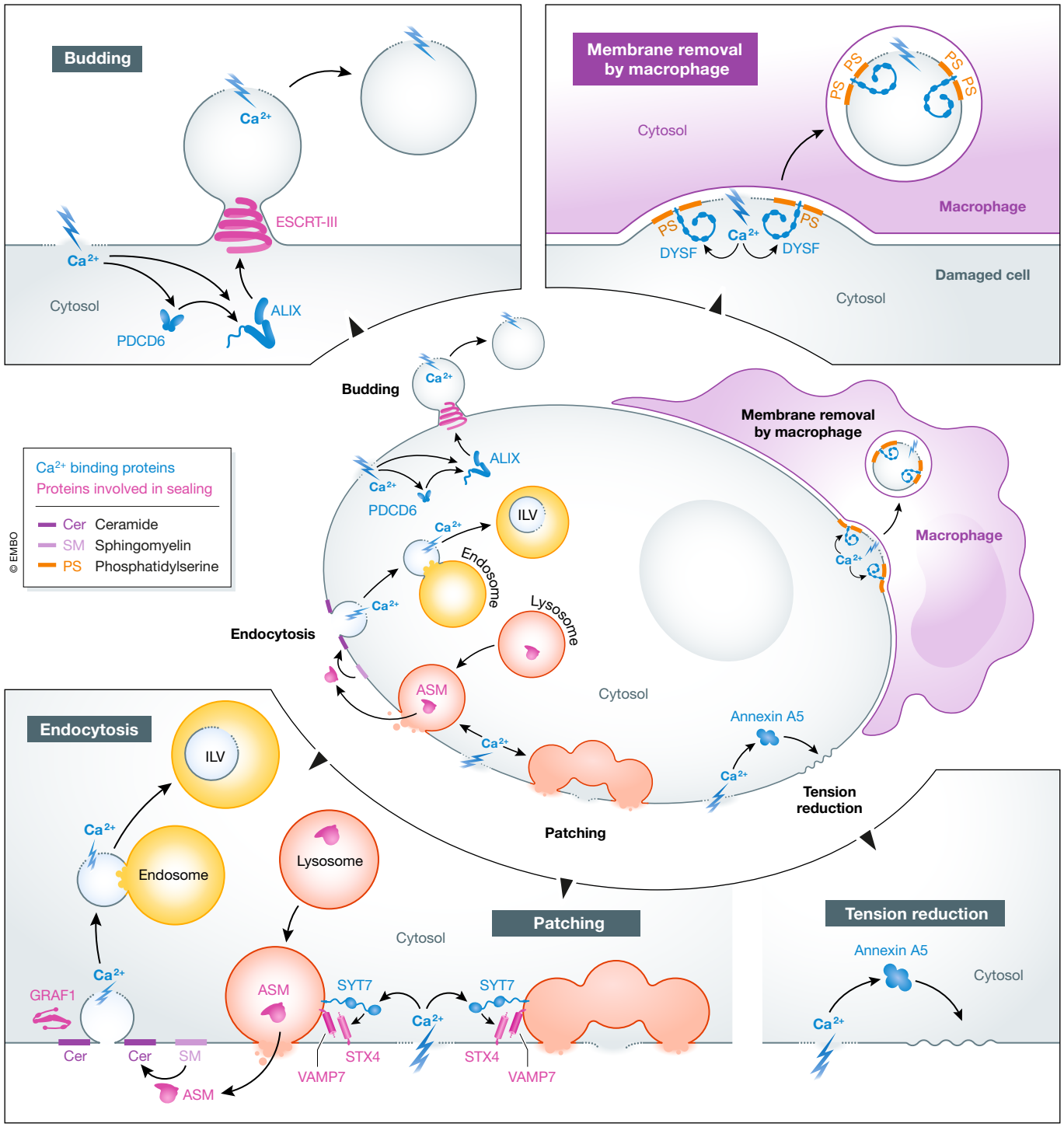

Figure 3. Plasma membrane repair.

Holes in the plasma membrane can be sealed by patching, endocytosis, budding, macrophage-mediated membrane removal or reduction in membrane tension. Ca^{2+} influx triggers all these processes. Ca^{2+} binding proteins are in green font, other proteins involved in sealing are in red font. Cer, ceramide; ILV, intraluminal vesicle; PS, phosphatidylserine.

pores around the damaged area, resulting in replacement of this area with a membrane patch from a large intracellular vesicle (such as a lysosome) has gained support (see Fig 1) (McNeil & Kirchhausen, 2005).

Later studies have shown that injury-dependent lysosome exocytosis after plasma membrane damage with Streptolysin O is followed by a burst of endocytosis of the damaged membrane area via flask-shaped invaginations known as caveolae (Idone *et al*,

2008) and that the pores end up in ILVs of late endosomes and lysosomes where they are degraded. A lysosomal enzyme, acid sphingomyelinase (ASM), provides the link between damage-induced lysosome exocytosis and caveolar endocytosis. When ASM is released from cells as lysosomes fuse with the plasma membrane, sphingomyelin in the extracellular leaflet of the plasma membrane is converted into ceramide, and this triggers rapid endocytosis via caveolae (Tam *et al,* 2010). There is also evidence that decreased plasma membrane tension triggers another type of endocytosis mediated by GRAF1 (Holst *et al,* 2017), a protein with a known involvement in muscle cell plasma membrane repair (Lenhart *et al,* 2015).

Plasma membrane damage induced by mechanical force, laser radiation, detergents or pore-forming toxins causes a rapid redistribution of ESCRT-I and -III proteins to the site of damage (Jimenez *et al,* 2014). This is accompanied by the accumulation of extracellular buds that are shed from the membrane. Depletion of ESCRT-III subunits or VPS4B prevents plasma membrane repair, and it is reasonable to conclude that ESCRT-III and VPS4B cooperate to mediate plasma membrane repair by promoting extracellular budding and shedding of plasma membrane domains containing the site of damage. Damage-induced ESCRT recruitment to the plasma membrane is Ca^{2+}-dependent, and the requirement of PDCD6, ALIX and Annexin A7 for ESCRT-III recruitment and plasma membrane repair suggests that these proteins could function as Ca^{2+} sensors that trigger ESCRT recruitment (Scheffer *et al,* 2014; Sonder *et al,* 2019). It still remains to be understood how ESCRT-III is recruited around the site of damage so that the damaged area becomes incorporated into the ESCRT-dependent bud.

Mouse perivascular cells devoid of Annexin A5 exhibit a severe defect in repair of their plasma membrane (Bouter *et al,* 2011), indicating a role for this annexin in plasma membrane repair. Annexin A5 assembles into local two-dimensional arrays at the plasma membrane in response to Ca^{2+} influx, and it has been proposed that these arrays prevent wound expansion and promote resealing by reducing cytoskeleton-induced membrane tension (Bouter *et al,* 2011).

Perhaps owing to their particular exposure to mechanical damage, muscle cells rely on an additional mechanism for repair of their plasma membrane, namely heterologous repair mediated by macrophages. Damage-induced influx of Ca^{2+} causes recruitment of the membrane protein Dysferlin (DYSF), whose cytosolic Ca^{2+} binding domain ensures clustering at the site of damage. A phosphatidylserine-binding motif in DYSF promotes accumulation of phosphatidylserine in the extracellular leaflet of the plasma membrane around the site of injury, marked by a "repair patch" consisting of proteins and lipids, and phosphatidylserine then causes recruitment of macrophages in an analogous way to apoptotic cells. However, whereas apoptotic cells, which contain phosphatidylserine all over their plasma membrane, are fully engulfed by macrophages, the macrophages only pinch off the damaged areas of the muscle cell plasma membrane (Middel *et al,* 2016). A potential source of DYSF and PS might be caveolae, which unfold on membrane stress and thus rapidly increase membrane surface (Sinha *et al,* 2011). It is interesting to note that DYSF interacts with MG53/TRIM72, another phosphatidylserine-binding protein involved in repair of the muscle cell plasma membrane (Cai *et al,* 2009). The proposed mechanism of TRIM72 is in fusion-mediated patching of holes in the plasma membrane, but its involvement in macrophage-mediated repair has not been clarified.

Repair of the damaged nuclear envelope

Because of its rigidity and large size, the nucleus is particularly prone to membrane damage when the cell moves through a confined space. In addition, reversible nuclear envelope ruptures are frequently detected in genetic disorders caused by mutations in proteins of the nuclear lamina, known as laminopathies, or in cancer cells (De Vos *et al,* 2011; Vargas *et al,* 2012; Denais *et al,* 2016; Raab *et al,* 2016; Earle *et al,* 2020). Upon rupture, uncoordinated mixing of nucleocytoplasmic components such as transcription factors, mRNA processing proteins, DNA damage repair factors and mitochondria and PML bodies occur and may affect a number of cellular functions (De Vos *et al,* 2011; Vargas *et al,* 2012).

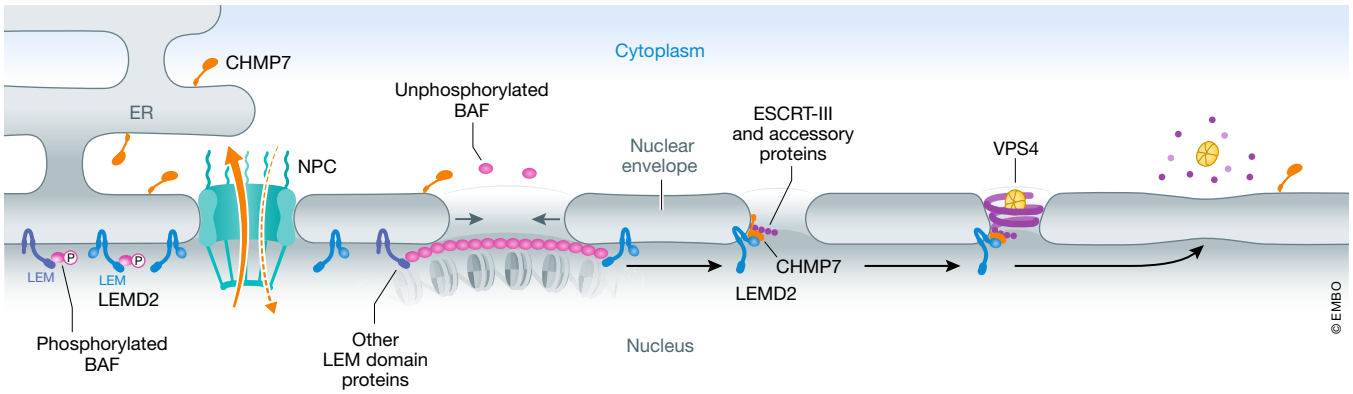

Figure 4. Nuclear envelope repair.

Under unperturbed conditions, the inner nuclear membrane protein LEMD2 and the endoplasmic reticulum-associated protein CHMP7 are spatially separated because CHMP7 is actively exported out of the nucleus through nuclear pore complexes (NPCs). Following rupture, unphosphorylated cytosolic BAF coats the exposed chromatin. Interaction of BAF with integral membrane LEM-domain proteins facilitates recruitment of nuclear membrane and decreases the size of the rupture. Subsequently, the CHMP7 and LEMD2 interaction promotes nucleation and polymerization of ESCRT-III, which together with the regulatory ATPase VPS4 further constricts the rupture and promotes sealing.

The exposure of genomic DNA to the cytosolic nuclease TREX1 causes accumulation of DNA damage and leads to reduced cell viability in laminopathies and senescence in non-transformed cell lines (Earle *et al,* 2020). Nuclear envelope ruptures at chromatin bridges and micronuclei lead to extensive DNA damage, kataegis, chromosome shattering and chromothripsis, supporting the generation of complex genomes in cancer cells (Maciejowski *et al,* 2015; Zhang *et al,* 2015; Umbreit *et al,* 2020). Along with these effects, prolonged exposure of DNA to the cytoplasm activates innate immunity and inflammatory responses dependent on the cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS) (Harding *et al,* 2017; Mackenzie *et al,* 2017).

In mammalian cells, repair of large ruptures first requires the mobilization of nuclear envelope membranes to coat the exposed chromatin. This is achieved through the action of barrier-to-autoantigen factor (BAF), a protein that exists in the cell as an unphosphorylated cytosolic pool, and as a phosphorylated nucleoplasmic pool which bridges the chromatin to the nuclear envelope. Upon nuclear envelope rupture, cytosolic BAF readily binds the exposed DNA and recruits membrane through its interaction with nuclear transmembrane proteins containing a LEM domain, such as LEMD2 (Halfmann *et al,* 2019; Young *et al,* 2020). BAF may in addition have a role in keeping the exposed chromatin compacted so that membrane coating and resealing are facilitated (Samwer *et al,* 2017; Penfield *et al,* 2018; Robijns *et al,* 2018). Subsequently, sealing depends on ESCRT-III (Denais *et al,* 2016; Raab *et al,* 2016), similarly to sealing of nascent nuclear envelopes during mitotic exit (Olmos *et al,* 2015; Vietri *et al,* 2015). The overall mechanism of sealing appears to be the same as with nascent nuclear envelopes, with CHMP7 playing an essential role as nuclear envelope integrity sensor and ESCRT-III recruiter (Fig 4). CHMP7 contains a nuclear export signal which ensures that CHMP7 is kept out from the nucleus at steady state (Thaller *et al,* 2019; Vietri *et al,* 2020b). However, whenever the integrity of the nuclear envelope is compromised, CHMP7 is recruited to the inner nuclear membrane by interacting with the inner nuclear membrane proteins LEMD2 and

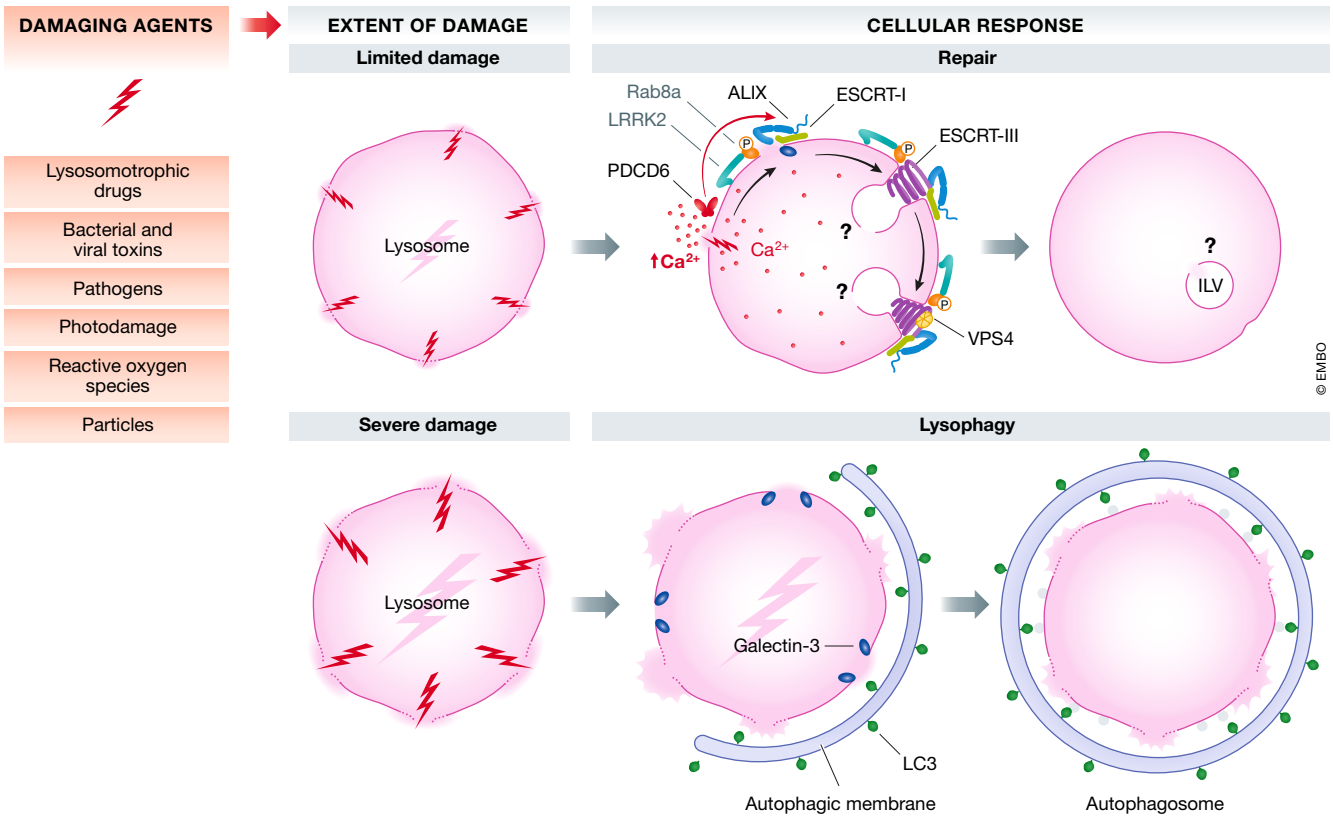

Figure 5. Lysosome repair.

Damaging agents such as lysosomotropic drugs, photodamage, pathogens etc. may inflict varying extent of lysosomal damage, leading to Ca^{2+} efflux from damaged lysosomes. The ESCRT machinery orchestrates repair of the limited lysosomal damage through multiple mechanisms in a coordinated manner. The increase in cytosolic Ca^{2+} is probably sensed by PDCD6 which in turn recruits the ESCRT-III binding protein ALIX. The ESCRT-I protein TSG101 and ALIX further recruit the ESCRT-III machinery together with VPS4 to seal the membrane lesions. Although Ca^{2+} efflux might provide an immediate signal for ESCRT-III recruitment, the β-galactoside sensor GAL3, which interacts with ALIX, is required for efficient recruitment of ALIX and ESCRT-III to the damaged lysosomes. This sealing might be accompanied by formation of ILVs containing the damage, similar to the process of endosomal ILV biogenesis. In macrophages, phagolysosome or lysosome damage triggers activation of the kinase LRRK2. Once activated, LRRK2 phosphorylates the small GTPase Rab8A, and ESCRT-III is recruited to mediate endolysosomal membrane repair in Ca^{2+}-dependent fashion. Severely damaged lysosomes are engulfed and degraded via lysophagy, initiated by β-galactoside sensors such as GAL3, which recruits LC3-containing autophagic membranes.

LEMD3. This translocation of CHMP7 is succeeded by ESCRT-III recruitment and membrane repair (Vietri *et al,* 2020b).

It is interesting to note that excessive CHMP7 and ESCRT-III recruitment, rather than promoting repair, causes aberrant folding of the nuclear envelope, accompanied by chromosome damage due to torsional stress. While such excessive ESCRT recruitment to the primary nucleus does not normally occur, it happens frequently to damaged micronuclei—single chromosomes enclosed by a micronuclear envelope. Thus, injury to the micronuclear envelope does not lead to repair but rather to catastrophic membrane rearrangements and chromosome damage (Vietri *et al,* 2020b).

Repair of the damaged lysosome membrane

Molecules, particles and microorganisms that are internalized into cells are harboured in organelles of the endocytic pathway, including endosomes, phagosomes, macropinosomes and lysosomes. All these organelles are exposed to membrane damage, and this is particularly the case with lysosomes, since these represent the terminal stations for many internalized cargoes and since their low intraluminal pH favours accumulation of weak bases that may cause membrane damage due to osmotic swelling.

Severely damaged lysosomes are removed by a selective autophagy process known as lysophagy, which entails sequestration of the damaged lysosome into an autophagosome, followed by fusion with a healthy lysosome in order to degrade the sequestered content (Hung *et al,* 2013; Maejima *et al,* 2013). However, more limited damage to the lysosome membrane can be repaired, and here, the ESCRT machinery plays a key role (Radulovic *et al,* 2018; Skowyra *et al,* 2018). Current evidence suggests that both Ca^{2+} flow from the damaged lysosome and cytosolic access to intraluminal β-galactoside sugars play roles in recruitment of the ESCRT machinery (Skowyra *et al,* 2018; Jia *et al,* 2020b) (Fig 5).

Like with damage to the plasma membrane, Ca^{2+} efflux from damaged lysosomes is accompanied by recruitment of the Ca^{2+} sensor PDCD6 (Skowyra *et al,* 2018), and several laboratories have reported an inhibitory effect of Ca^{2+} chelators on ESCRT recruitment to damaged lysosomes (Skowyra *et al,* 2018; Jia *et al,* 2020b). Indeed, the Ca^{2+}- and PDCD6-binding protein ALIX plays an important role in ESCRT-III recruitment to damaged lysosomes. Surprisingly, however, depletion of ALIX alone has minimal effect on ESCRT-III recruitment whereas its co-depletion with the ESCRT-I subunit TSG101 (which has a moderate effect on ESCRT-III recruitment by itself) strongly prevents ESCRT-III recruitment (Radulovic *et al,* 2018; Skowyra *et al,* 2018). This indicates that the mechanism of ESCRT-III recruitment is more complex than that to the damaged plasma membrane and may involve additional factors. One of these is GAL3, which interacts with ALIX and is required for efficient recruitment of ALIX and ESCRT-III to damaged lysosomes. Whereas Ca^{2+} efflux might provide an immediate signal for ESCRT-III recruitment, GAL3 could provide a later and more sustained signal, and GAL3 has the additional function of promoting lysophagy in case of more severe lysosome damage (Jia *et al,* 2020b).

An additional factor that determines the fate of damaged phagolysosomes and lysosomes in macrophages is the Parkinson's disease-related leucine-rich repeat kinase 2 (LRRK2). LRRK2 is activated upon lysosome damage, which then triggers further recruitment of the small GTPase Rab8A and subsequently the ESCRT-III protein CHMP4B to damaged organelles (Herbst *et al,* 2020). Whether this cascade also mediates repair in other cell types or is specific for macrophages requires further investigation.

It is not yet known how the ESCRT machinery functions to seal holes in the lysosome membrane, but from the known topologies of other ESCRT-dependent processes (Vietri *et al,* 2020a) it is tempting to speculate that it functions by shedding of vesicles containing membrane damage into the lumen of the lysosome.

Even though leakiness of lysosomes is generally thought to be harmful to the cell, a spatially and temporally controlled leakage of lysosomes is actually required for accurate chromosome segregation in normal mammalian cell division (Hamalisto *et al,* 2020). During metaphase, cathepsin B leaks out of chromosome-proximal lysosomes to cleave a small subset of histone H3 in order to maintain chromosome fidelity in mitosis. It will be interesting to understand how this selective lysosome permeabilization is initiated and terminated.

Conclusions and perspectives

There is strong evidence that multiple mechanisms have evolved to seal holes in membranes, and there may be good reasons why separate mechanisms exist. Firstly, because membrane integrity is so crucial for cellular viability and functions, the existence of multiple sealing mechanisms could ensure successful sealing even if one mechanism fails. Secondly, the different mechanisms are optimized for sealing of different types of holes. The smallest holes with a diameter of a few nm may be self-sealed, or self-sealing could be promoted by proteins that reduce membrane tension, such as annexins. Holes with a diameter of 50–100 nm are typically sealed by endocytosis or ESCRT-mediated outward scission, whereas the largest holes of more than 100 nm could require patching by intracellular membranes, or engulfment by neighbouring cells. It is also plausible that additional mechanisms of membrane sealing exist, which have not been characterized yet. For instance, regulated lipid synthesis has been proposed as a mechanism (Penfield *et al,* 2020), and it is also possible to envisage intermembrane lipid transfer to seal holes, possibly mediated via membrane contact sites (Elbaz & Schuldiner, 2011; Raiborg *et al,* 2015).

Although we here have focused on sealing of the plasma membrane, the nuclear envelope, lysosomes and autophagosomes, the integrity of other cellular membranes is certainly also critical in biology and health. For example, permeabilization of mitochondrial membranes is known as a major trigger of cell death (Kroemer & Reed, 2000). It will therefore be exciting to learn whether there are cellular mechanisms that seal such membranes as well.

The importance of cellular membrane integrity is underscored by the many genetic diseases associated with compromised integrity of the plasma membrane and the nuclear envelope in particular. Mutations that either compromise membrane integrity as such or affect membrane repair systems are typically associated with diseases that involve reduced functions of cells exposed to mechanical damage, including skeletal and cardiac muscle cells. Long-lived cells with very limited capacity for self-renewal, such as neurons, are also vulnerable to membrane damage, and this is reflected by the number of diseases of the peripheral and central nervous system associated with compromised membrane integrity (Chi *et al,* 2009).

While repair of damaged membranes provides crucial cellular defence against viruses and microorganisms and their pathogenicity

factors (Etxaniz *et al,* 2018), some microorganisms can subvert cellular membrane repair mechanisms for their entry or replication. For instance, *Trypanozoma cruzi* hijacks the ASM-mediated membrane repair pathway for its invasion into cells (Fernandes *et al,* 2011), whereas *Coxiella burnetii* takes advantage of ESCRT-mediated repair of the bacterial vacuole for its intravacuolar replication (Radulovic *et al,* 2018). This means that, under some conditions, membrane sealing can be a disadvantage for the host.

The importance of membrane sealing for cell viability and organismal health, and its exploitation by certain pathogens, makes it relevant to ask whether manipulation of membrane sealing could have therapeutic applications. Selective prevention of membrane sealing could be beneficial for killing cancer cells or preventing intracellular replication of pathogens that rely on an intact vacuole. Conversely, stimulating membrane sealing could ameliorate symptoms in patients with genetic diseases associated with membrane fragility of dysfunctional repair. While prevention of membrane sealing could potentially be achieved with small-molecule inhibitors of sealing factors, stimulating sealing would probably require gene therapeutic approaches. A major obstacle to these approaches is the complexity of cellular membrane sealing reactions, and their reliance on proteins that often have multiple cellular functions. Nevertheless, as we learn more and more about the mechanisms of cellular membrane sealing, therapies directed against membrane sealing could soon become a reality.

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Conflict of interest

The authors declare that they have no conflict of interest.

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