Review

Molecular Detection of Membrane Damage in Mammalian Cells

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Molecular Detection of Membrane Damage in Mammalian Cells

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Abstract

Mammalian cells contain numerous membrane-bound organelles, among which endosomes serve as the initial destination for endocytosed materials. Drugs and pathogens are internalized by cells and transported to endosomes or phagosomes, and are subsequently delivered to lysosomes for degradation. Therefore, internalized drugs must escape from endosomes into the cytosol before lysosomal degradation occurs. However, endosomal escape is often inefficient in artificial drug delivery systems (DDSs). In contrast, many pathogens such as bacteria are phagocytosed and subsequently escape into the cytosol where they proliferate successfully. Studies on bacterial phagosomal escape have revealed molecular mechanisms by which host cells detect damage to organelle membrane. These host cellular machineries for sensing membrane damage can also detect membrane damage caused by artificial drugs. In this review, we summarize current knowledge of the cellular machinery involved in sensing membrane damage, including galectins, ESCRT complexes, sphingomyelin, stress granules, phosphatidylinositol 4-phosphate (PI4P) at membrane contact sites, and annexins. Although the aim of this review is to identify the molecules involved in endosomal membrane damage, many of these molecules were initially discovered through studies of bacterial infection and damage to the plasma membrane or lysosomes. Research on membrane damage not only advances our understanding of cellular responses to organelle damage, but also provides insights into the toxicity induced by inorganic materials and contributes to the rational design of more effective DDSs.

Keywords: membrane damage; galectin; ESCRT; sphingomyelin; secretory MVBs

1. Introduction

Mammalian cells internalize nutrients, solutes, growth factors, or hormones that bind to specific receptors on the plasma membrane through endocytosis. These cargos are internalized together with their receptors. Several endocytic pathways have been described, including clathrin-mediated endocytosis (CME), fast endophilin-mediated endocytosis (clathrin-independent but dynamin-dependent), clathrin-independent carrier (CLIC)/glycosylphosphatidylinositol-anchored protein enriched early endocytic compartment (GEEC) endocytosis, macropinocytosis, phagocytosis, and caveolin-dependent endocytosis [1–3]. In addition, there may be uncharacterized endocytic pathways. Solutes that do not bind to specific receptors can also be internalized through these pathways together with extracellular fluid by bulk flow. Cargos can be internalized through different endocytic pathways depending on their size [3–5]. Although pharmacological inhibitors of these pathways are not entirely specific, cargos smaller than ~200nm can be internalized through CME, FEME, caveolin-dependent endocytosis, or CLIC/GEEC pathways. Cargos with ~250-500 nm can be internalized by macropinocytosis or phagocytosis. Large cargos such as bacteria or nanoparticles larger than ~500 nm are typically internalized by Phagocytosis. In phagocytosis, the space between the membrane and the cargo is relatively small, whereas macropinocytosis involves large membrane ruffles that enclose extracellular fluid with a larger space between the membrane and the cargo [6,7]. Through these endocytic pathways, most of the cargoes are encapsulated by transport career such as vesicles,tubules,

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macropinosomes or phagosomes that subsequently fuse with early endosomes and mature into lysosomes [8,9].

Early endosomes function as sorting hubs that direct cargos to their respective destinations. Endosomal maturation has been extensively reviewed elsewhere [8–13]. Briefly, following internalization, early endosomes mature into late endosomes/multi-vesicular bodies (MVBs) and subsequently into lysosomes for degradation. In parallel, specific cargos are sorted within early endosomes and transported to recycling endosomes for return to the plasma membrane or via retrograde transport pathways to the Golgi apparatus. Cargos that reach the Golgi apparatus are either recycled back to endosomes or further transported retrogradely to the endoplasmic reticulum (ER). The bulk flow route is generally considered to represent the degradation pathway to lysosomes, as early endosomes matures into late endosomes rather than delivering cargos to pre-existing late endosomes [12,14].

Drug Delivery Systems (DDSs) designed to promote the transport of drugs from endosomes into the cytosol have been extensively studied to prevent lysosomal degradation of therapeutic cargos. Strategies of endosomal escape used by pathogens are often applied to DDS design. Several pathogenic bacteria, such as *Shigella flexneri* (*S. flexneri*) and *Listeria monocytogenes* (*L. monocytogenes*) are well known to escape from phagosomes [15,16]. In the case of *L. monocytogenes,* pore forming toxin (PFT) whose activity is dependent on acidic pH [17,18]. Along endocytic pathway, early endosomes are acidified to a pH of approximately 6.2-6.8, followed by further acidification to pH 5.2-6.0 in late endosomes, and finally to pH 4.5-4.8 in lysosomes, where proteins are degraded [19–22]. PFT activated by acidic pH is thought to promote bacterial replication in the cytosol while limiting host cell death, compared with pH-independent PFT activity. Therefore, endosomal escape could be less toxic to host cells than damaging plasma membrane in this case [23,24]. Consequently, sensitivity to acidic pH has frequently been incorporated into the design of DDSs as a trigger for drug release, including pH-sensitive amphiphilic polymers or peptides [23–25].

Studies of membrane damage can be also beneficial to understand the toxicity of DDS, nanoparticles or artificial treatments to cells. MVBs can be damaged by internalized wear debris derived from joint replacement [26](see section 2.7.2). Early endosomes can be damaged by osmotic shock [27], or by magnetic nanoparticles under an external magnetic field [28]. Recycling endosomes can be damaged by an ionophore Monensin [29]. Recycling endosomes have been also implicated as sites of endosomal escape induced by lipid nanoparticles [30]. Moreover, Rab GTPases associated with recycling endosomes, such as Rab11, 8 and 35, play important roles in phagosomal escape by *S. flexneri* [31–34]. These findings suggest that recycling endosomes may play critical roles in endosomal membrane disruption.

Membrane damage can be detected by host cell-derived sensor molecules. These molecules often participate in membrane repair processes and ultimately trigger autophagy to eliminate damaged organelles. Although many of these sensing mechanisms were initially identified through studies of bacterial infection, the same host cell molecules are also capable of detecting “sterile” membrane damage, such as lysosomal membrane damage induced by L-leucyl-L-leucine methyl ester (LLOMe). As a non-bacterial damaging agent, LLOMe is widely used as a tool to induce lysosomal membrane damage in experimental settings [35].

In this review, we describe molecules implicated in cellular responses to membrane damage with the aim of identifying those that may be capable of detecting endosomal escape. However, many of these molecules were originally identified through studies of damage to the plasma membrane, phagosomes or lysosomes, and are therefore not specific to damaged endosomes. There are several differences between endosomal escape induced by DDS and other types of membrane damage. Whereas damage to the plasma membrane can be highly toxic to cells, damage to endosomal membrane may be less detrimental. Whereas bacteria secrete biological factors that regulate host cell activities [36], DDS do not secrete such factors. In addition, whereas lysosomal damage releases highly acidic contents into the cytosol, the contents released form damaged endosomes may be less acidic than those from lysosomes. To describe the characteristics of these sensor molecules, we

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discuss how they were initially identified, including studies of phagosomes in bacterial infection. We also describe molecules involved in sensing "sterile" membrane damage caused by laser-induced plasma membrane injury or lysosomal damage induced by LLOMe. These studies provide insights into cellular responses when DDSs are introduced into cells.

We focus on the early phase of membrane damage detection (typically within 1-30 min), rather than on later cellular responses such as autophagy or inflammation. Autophagy has been extensively reviewed elsewhere [37,38]. We compiled the reported recruitment times of these molecules to damaged membranes from the literature (see Table 1). Although advances in imaging technologies may enable the detection of membrane damage at much earlier time points in the future, side-by-side comparison of recruitment timing based on currently available data provides a useful framework for understanding how cells sense damaged organelles and the subsequent cellular events that follow membrane damage.

2. Molecular Sensors of Membrane Damage in Mammalian Cells

2.1. Galectins

2.1.1. Galectin-3

During phagosomal escape of *S. flexneri* in HeLa, J774 macrophage, or CHO cells, Galectin-3 (Gal-3) is recruited to the phagosomal membrane surrounding the bacterium [39,40]. Galectins are glycan-binding proteins, and Gal-3 preferentially binds to LacNAc (Galβ(1-4)GlcNAc), which is present internally in glycan residues [41,42]. Gal-3 is recruited to the phagosomal membrane, encapsulating the bacterium as well as tubular structures connected to the limiting membrane of phagosomes. The glycan-binding ability of Gal-3 is required for this localization, suggesting that Gal-3 recognizes glycans exposed on the internal leaflet of damaged phagosomal membranes rather than lipopolysaccharide (LPS) present on cell wall of *S. flexneri* [40]. In addition to bacteria-containing phagosomes, Gal-3 is also recruited to "sterile" membrane damage in endosomes or lysosomes. For example, Gal-3 is recruited to damaged phagosomes induced by internalized 3 μm latex beads coated with cationic lipid transfection reagents [43]. Gal-3 is also recruited to early endosomes damaged by 20 nm magnetic nanoparticles under an external magnetic field [28]. Furthermore, Gal-3 accumulates on lysosomes damaged by L-leucyl-L-leucine methyl ester (LLOMe) [44]. LLOMe destabilizes the lysosomal lipid bilayer by forming a polymer from a dipeptide of Leucine in a Cathepsin C-dependent manner [35].

Gal-3 colocalizes with internalized *S. flexneri* together with ubiquitin, p62 and LC3 [39]. Autophagy is a process in which cytosolic components are sequestered and degraded within double-membrane structures marked by lipidated LC3, and it plays crucial role in preventing *Shigella* proliferation [45]. p62 is an autophagy adaptor that binds polyubiquitin chains and links ubiquitinated cargos to LC3 [46,47].

Upon LLOMe treatment, K48- and K63-linked ubiquitin chains are conjugated to damaged lysosomes, indicating that ubiquitination occurs to damaged organelles under these conditions [48]. Screening of E2 ubiquitin-conjugating enzymes identified UBE2QL1, and both UBE2QL1 and K48- and K63-linked ubiquitin signals are detected 30-60 min after LLOMe treatment. Depletion of UBE2QL1 abolishes ubiquitin signals as well as the recruitment of LC3 and p97, which are required for the clearance of damaged lysosomes [48,49]. Interestingly, Gal-3 recruitment to damaged lysosomes is enhanced upon UBE2QL1 depletion, indicating that Gal-3 is recruited independently of ubiquitination. However, Gal-3 accumulation is indirectly influenced by impaired clearance of damaged lysosomes.

S. flexneri can be directly ubiquitinated on its LPS by the host E3 ligase RNF213 at approximately 2 h post-infection, leading to the induction of autophagy [50,51]. In *Mycobacterium tuberculosis* (*Mtb*), bacterial ubiquitination is likewise mediated by E3 ligases, including Parkin within ~4 h, and Smad ubiquitination regulatory factor 1 (Smurf1) within ~15 hours after infection [52,53]. In addition, the

 doi:10.20944/preprints202509.2282.v2

E3 ligase Leucine-rich repeat and sterile alpha motif-containing protein 1 (LRSAM1) is recruited to internalized *Salmonella enterica serovar Typhimurium* (*S. Typhimurium*) and *S. flexneri* within ~45 min [54]. Although ubiquitination of cytosolic bacteria plays an essential role in triggering antibacterial autophagy, Gal-3 recruitment occurs much earlier, within ~10 min, preceding detectable bacterial ubiquitination (see Table 1). These observations indicate that host cell detection of damaged organelles occurs prior to bacterial ubiquitination during the early stages of host defense.

Gal-3 can promote autophagy through interactions with components of the autophagy machinery. Gal-3 interacts with Tripartite motif-containing Protein 16 (TRIM16) which in turn recruits ATG16L to damaged lysosomes [55]. ATG16L is a core component of the autophagy machinery and is recruited to ubiquitin-positive *Salmonella* as well as ubiquitin-positive latex-beads in ubiquitin- and FIP200-dependent manner [43]. ATG16L further determines the site of LC3 lipidation and localization [56]. Depletion of TRIM16 inhibits ubiquitination of damaged lysosomes and partially reduces LC3 recruitment [55]. Similarly, depletion of Gal-3 partially decreases LC3 recruitment following LLOMe treatment [57]. Consistent with these cellular observations, Gal-3 KO mice show increased susceptibility to infection by *Mtb* [55]. Together, these findings indicate that Gal-3 and TRIM16 contribute, at least in part, to LC3 recruitment and promote autophagic clearance of damaged organelles.

However, autophagy can be triggered by multiple distinct signals, as described in Sections 2.2.3, 2.3.2 and 2.5. In addition to its pro-autophagic roles, depletion of Gal-3 has also been reported to enhance autophagy. Deletion of Gal-3 resulted in increased LC3 recruitment to phagosomes in *L. monocytogenes*-infected macrophages [58]. These findings suggest that Gal-3 may function to suppress LC3 recruitment to phagosomes, or alternatively, that depletion of Gal-3 impairs phagosomal membrane repair, thereby leading to enhanced LC3 recruitment [58]. Thus, whether Gal-3 inhibits or promotes antibacterial autophagy is likely context dependent and may vary according to the invading bacterium, the type of membrane-damaging agent, the cell type, and the presence of compensatory pathways activated upon Gal-3 depletion.

2.1.2. Galectin-8, 9

Although Gal-3, -8, and -9 were recruited to Salmonella-containing Vacuoles (SCVs) at 1 hour after infection with *S. Typhimurium* in HeLa cells, depletion of only Gal-8 increased proliferation of *S. Typhimurium* [59], suggesting that Gal-3 and Gal-9 are not directly involved in inhibiting *S. Typhimurium* proliferation. Gal-8 binds to LacNAc in a manner similar to Gal-3 [42], but preferentially interact with the autophagy adaptor NDP-52 rather than p62 [60]. Consistently, depletion of NDP-52 also increased *S. Typhimurium* proliferation [59], supporting roles for Gal-8 and NDP-52 in antibacterial activity against *S. Typhimurium.* Notably, after 4 h post-infection, Gal-8-independent recruitment of NDP-52 was observed, indicating that Gal-8 is an early detector for intracellular pathogens.

Gal-9 has been reported to be preferentially recruited to damaged endosomes and lysosomes by damaging agents, such as chloroquine and siramesine, to a greater extent than Gal-3 or Gal-8 [61]. Gal-9 has been utilized as a readout in high-throughput screening to identify efficient lipid nanoparticle formulations [62]. In cells, depletion of Gal-9 resulted in reduced ubiquitination of damaged lysosomes [63]. Upon LLOMe treatment, Gal-9 interacts with the deubiquitinating enzyme, USP9X, thereby inhibiting deubiquitination and promoting downstream ubiquitin-dependent processes, including autophagy [63]. Consistently, Gal-9 depletion suppressed lysosomal ubiquitination and inhibited LC3 lipidation following LLOMe treatment [63].

Although Gal-9 has been used as a marker to detect endosomal escape, it may also influence immune function. Gal-9 is expressed in a wide variety of immune cells, and soluble Gal-9 binding to Tim-3 on the surface of $CD4^+$ T cells suppresses T cell activity [64]. Gal-9-Tim-3 interaction has been reported to downregulate coreceptors on $CD4^+$ T cells, and to upregulates p21, thereby inhibiting HIV infection in these cells. In contrast, Gal-9 binding to secreted protein disulfide isomerase (PDI) on the surface of $CD4^+$ T cells enhances HIV infection in resting $CD4^+$ T cells [65]. These findings suggest

 doi:10.20944/preprints202509.2282.v2

that alterations in immune responses associated with Gal-9 expression should be taken into consideration when immune cells are employed as experimental systems.

2.2. ESCRTs

2.2.1. Overview of ESCRT Proteins

Endosomal sorting complexes required for transport (ESCRT) proteins have been identified as class E vacuolar protein sorting (Vps) proteins, including ESCRT-0, I, II, III, and the Vps4 ATPase [66–71]. Studies on ESCRT proteins in the trafficking of the epidermal growth factor receptor (EGFR), a well-known receptor tyrosine kinase [72–74], have deepened our understanding of the roles of the ESCRT complexes. Upon EGF binding, EGFR is ubiquitinated [75–79], and Hrs and STAM complexes and ESCRT-0 components bind to the ubiquitinated EGFR and transfer EGFR to ESCRT-I, II, and III, inducing inward budding of the limiting membrane to produce intraluminal vesicles (ILVs) that form [11,80,81]. Through inward budding, the phosphorylated cytosolic region of EGFR—which binds signaling complexes such as Grb2 [82,83]—is incorporated into ILVs and degraded in lysosomes together with the luminal region of EGFR [73,74]. CHMP4A and CHMP4B, which are ESCRT-III proteins, form filaments [84], and ESCRT-III mediates membrane scission to form ILVs [85,86]. ESCRT-III assembles at the bud neck and constricts the membrane with Vps4 to sever the remaining membrane [87,88]. Thus, ESCRT proteins form vesicles by pushing the membrane from the cytosol into the lumen, which is an inverse mechanism compared to coat proteins such as clathrin or COPI, which invaginate vesicles by pulling the membrane from the lumen to the cytosol. At present, ESCRT proteins are known to have many functions beyond ILV formation, and their unique membrane scission activities plays important roles in diverse cellular events [81].

2.2.2. ESCRT in Plasma Membrane Damage

When the plasma membrane is damaged by digitonin, streptolysin O, or UV laser ablation, CHMP4B—an ESCRT-III component—is recruited to the wound site prior to the initiation of membrane repair [89]. Depletion of CHMP4B decreases cell survival following wounding, indicating that the ESCRT machinery plays a critical role in plasma membrane repair [89]. It has been proposed that ESCRT-III and Vps4 function in membrane repair by constricting intact membrane regions, thereby shedding damaged membrane portions as extracellular vesicles [89].

How cells detect plasma membrane damage and what initial events precede CHMP4B recruitment, have been actively investigated. The ESCRT subunits involved in plasma membrane damage repair are not identical to those functioning in EGFR sorting into ILVs. While ALG2, TSG101, ALIX and CHMP4B are recruited to the plasma membrane in Ca^{2+} -dependent manner, ESCRT-0 components Hrs and STAM, the ESCRT-I subunit Vps37, and the ESCRT-II subunit Vps25 are not detected [89,90]. ALIX binds both TSG101 and CHMP4B, thereby bridging Tsg101/ESCRT-I and ESCRT-III [91–94]. ALIX was originally identified as a binding partner of Apoptosis-linked-gene 2 (ALG2), and was therefore named ALG-2 interacting protein 1 or X (AIP1/ALIX) [95,96]. Under steady-state conditions, ALIX localizes to MVBs in a manner dependent on lysobisphosphatidic acid (LBPA) [97–99]. ALIX binds ALG2 in a Ca^{2+}-dependent manner, and ALG-2 itself exhibits oscillatory localization between the cytosol and ER-exit sites in response to Ca^{2+} [95,96,100,101]. Upon plasma membrane injury, ALIX is recruited to plasma membrane lesions independently of MVBs [89,90]. Notably, ALG2 and ALIX are recruited earlier than CHMP4B and Vps4B to damaged sites, and depletion of ALG2 impairs the recruitment of ALIX, CHMP4B and Vps4B to the plasma membrane [90], suggesting that Ca^{2+}-dependent binding of ALG2 to damaged membrane is critical for the initial detection of plasma membrane injury and subsequent recruitment of ESCRT components. A recent study further demonstrated that ALG2 binds Annexin A7, another Ca^{2+} -dependent membrane-binding protein [102](see Section 2.7). Annexin A7 knockout delays ALG2 recruitment to the plasma membrane lesions and inhibits CHMP4B accumulation required for membrane shedding, suggesting that Annexin A7 and ALG2 cooperatively detect plasma membrane injury in a Ca^{2+} -dependent

 doi:10.20944/preprints202509.2282.v2

manner and subsequently recruit ESCRT components for plasma membrane repair [102]. Ubiquitin is also observed at damaged plasma membrane; however, its accumulation occurs after CHMP4B recruitment. Whereas CHMP4B is detected within 10-60 sec following injury, ubiquitination becomes evident only after approximately 6 min [89], indicating that ubiquitin conjugation is a downstream event relative to ESCRT assembly.

Recent studies have further shown that ALIX is not always required for plasma membrane repair, whereas TSG101 plays an essential role [103]. Furthermore, other Ca^{2+}-dependent proteins, including IQGAP1 (see Section 2.6.2), have been implicated in membrane repair. Collectively, these findings suggest that multiple, potentially parallel pathways contribute to plasma membrane repair.

2.2.3. ESCRT in Lysosomal Membrane Damage

ESCRT proteins are also involved in the repair of damaged lysosomal membranes [104,105]. The lysosome-damaging agents, LLOMe, and glycyl-L-phenylalanine 2-naphthylamide (GPN)—a substrate of Cathepsin C that induces osmotic lysis of lysosomes [106–108]—have been shown to recruit the ESCRT components ALIX and CHMP4B to damaged lysosomes. Depletion of ALIX and TSG101 impairs CHMP4B recruitment and compromises lysosomal repair [57,104,105]. These findings indicate that, similar to plasma membrane lesions, ALIX, TSG101, and ESCRT-III are recruited to damaged lysosomes and play critical roles in lysosomal repair.

Ca^{2+} are also involved in this process and facilitate the recruitment of ALIX and CHMP4B [105]. Ca^{2+} is required for the recruitment of ALIX and CHMP4A to damaged lysosomes within 10-15 minutes after lysosomal injury [57]. At later time points, approximately 30 min after damage, Gal-3 depletion reduces ALIX and CHMP4B recruitment to damaged lysosomes [57], suggesting Gal-3 contributes to CHMP4B recruitment during the later phase of lysosomal repair. However, CHMP4B recruitment precedes of Gal-3 accumulation [104,105], and depletion of ALIX and TSG101 does not inhibit Gal-3 recruitment within 10 min after LLOMe treatment [57,104]. ESCRT machinery is also preferentially recruited to small membrane lesions [105]. These observations suggest that ESCRT is recruited during the early phase of lysosomal damage in a Ca^{2+}-dependent manner, followed by Gal-3 recruitment. Gal-3 is known to interact with ALIX [109,110], and forms a complex with ALIX and CHMP4B at 30 minutes after lysosomal damage [57]. Thus, while the initial recruitment of ALIX is primarily Ca^{2+}-dependent, ALIX can be recruited during later stages through both Ca^{2+}-dependent and Gal-3-mediated mechanisms. Recruitment of ALIX and CHMP4B precedes that of Gal-3 and lysosomal ubiquitination [104,105]. Gal-3 depletion compromises the recruitment of LC3 as well as the autophagy machinery components ATG13 and ATG16L, supporting a role for Gal-3 in promoting autophagy of damaged lysosomes [57].

Lysosomes damaged by LLOMe or silica nanoparticle recruit IST1 and SPG20 [111]. IST1 is an ESCRT-III-like protein containing a CHMP-like domain [112]. IST1 binds to ALG-2 in a Ca^{2+}-dependent manner and forms a complex with CHMP1A and CHMP1B [112], suggesting that IST1 acts as an additional Ca^{2+}-dependent adaptor linking ALG-2 to ESCRT-III. Although IST1 binds to SPG20, deletion of the IST1-binding domain in SPG20 only partially reduces SPG20 recruitment to damaged lysosomes [111]. SPG20 has an SC domain that binds to loosely packed lipids generated by lipid peroxidation. Lipid peroxidation induces SPG20 recruitment to damaged lysosomes, whereas Ca^{2+} release without lipid-packing defects—induced by ML-SA1, an agonist of lysosomal Ca^{2+} channel TRPML1— does not recruit SPG20, even though IST1 can be recruited under these conditions [111]. These findings indicate that SPG20 can recognize damaged membranes directly, as in addition to being recruited via IST1. SPG20 forms a complex with ubiquitin ligase ITCH, which is also recruited to damaged lysosomes. Depletion of ITCH reduces K63-linked ubiquitination on lysosomes, leading to decreased LC3 recruitment and reduced cell survival [111]. These observations implicate a lipid damage-dependent pathway of lysosomal ubiquitination in lysophagy.

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2.2.4. ESCRT in Endosomal Membrane Damage

Recruitment of ALIX, CHMP4B, and CHMP1B has been observed at damaged early endosomes positive for early endosome markers EEA1 and Rab5, and the depletion of ALIX and TSG101 inhibits CHMP4B recruitment [27]. Rupture of early endosomes can be induced by hypertonic shock, which promotes water efflux to extracellular space, and a decrease in membrane tension has been detected using the tension probe, FliptR, whose fluorescent lifetime decreases when membrane tension is reduced [113,114]. Reduced membrane tension in early endosomes correlates with the recruitment of ESCRT proteins [27]. A decrease in membrane tension can also be caused by the fusion of many endocytic vesicles with endosomes following EGF treatment, which likewise triggers ESCRT recruitment [27].

Membrane damage in early endosomes can also be induced by the internalization of 20 nm of magnetic nanoparticles under a 100 mT of magnetic field for 5 min [28]. Under these conditions, recruitment of Gal-3 together with CHMP4B was observed to EEA1-positive early endosomes, whereas LC3 recruitment was not detected [28]. These observations are consistent with the idea that the ESCRT machinery is recruited to small membrane lesions during the early phase of damage, and that once membrane integrity is restored, LC3 recruitment is not required.

2.2.5. ESCRT in Bacteria-Containing Vacuoles

Internalized bacteria often form specialized intracellular compartments for proliferation, and ESCRT proteins are recruited to these bacteria-containing vacuoles. *Coxiella burnetii*—a Gram-negative bacterium that proliferates within lysosomes—also induces ESCRT recruitment to lysosomes [104,105]. *Salmonella*-containing vacuoles (SCVs) are specialized organelles in which *Salmonella* proliferates without escaping into the cytosol. Depletion of CHMP3, an ESCRT-III component, induces enlargement of SCVs and significantly increases cytosolic proliferation of *S. Typhimurium* [115]. These findings suggest that ESCRT proteins play important roles in controlling the size and morphology of SCVs and in restricting bacterial proliferation.

The deubiquitination enzyme USP8 removes ubiquitin from ESCRT-III components such as CHMP2B and CHMP4 [116]. Depletion of USP8 increases the levels of CHMP4B that colocalize with intracellular *Mtb* and enhances autophagic responses that restrict *Mtb* proliferation [117]. In LLOMe-treated cells, USP8 depletion also causes CHMP4B to persist on damaged lysosomes even after wash out; however, recruitment of Vps4 is impaired, suggesting that deubiquitination of CHMP4B is required for Vps4 recruitment. USP8 depletion further enhances recruitment of autophagy receptors such as SQSTM1/p62, indicating that defects of ESCRT-mediated membrane repair promotes autophagy [117].

2.3. Sphingomyelin

2.3.1. Sphingomyelin Exposure in Phagosomal Escape

Sphingomyelin is an important component of lipid rafts. Lipid rafts are membrane microdomains originally characterized as detergent-resistant membranes (DRMs) and are sensitive to methyl-β-cyclodextrin (MβCD), which depletes cholesterol and disrupts liquid-ordered phase of the membrane [118,119]. Sphingomyelin is synthesized from ceramide in the lumen of the Golgi apparatus [120] and is subsequently transported to the plasma membrane, endosomes, and lysosomes [121–124]. Sphingomyelin predominantly localizes to the luminal leaflet of intracellular organelles or the outer leaflet of the plasma membrane [121].

Phagosomal membrane damage induced by *S. Typhimurium*, *S. flexneri*, *L. monocytogenes*, and *Streptococcus pyogenes* exposes sphingomyelin to the cytosolic side of the membrane [125]. Cytosolic exposure of sphingomyelin is detected by the C-terminal region of lysenin, a sphingomyelin-binding proteinaceous toxin isolated from the earthworm *Eisenia foetida* [126]. Recruitment of lysenin to *S. Typhimurium*- and *S. flexneri*- containing phagosomes precedes that of Gal-8. Electron microscopy

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revealed that damaged membranes positive only for lysenin contain gaps of approximately 100–200 nm, whereas membranes positive for both lysenin and Gal-8 display larger discontinuities and no longer enclose *S. Typhimurium*. These observations suggest that exposure of sphingomyelin to the cytosol occurs at an earlier stage than galectin recruitment during phagosomal membrane damage [125].

TECPR1 was identified as an endogenous receptor for *sphingomyelin* exposed to the cytosol by sphingomyelin-containing liposomes [127]. TECPR1 localized to phagosomes containing *S. flexneri, S. Typhimurium*, *L. monocytogenes,* as well as endosomes or lysosomes damaged by osmotic shock or LLOMe [127]. TECPR1 has been shown to bind ATG5 and to promote LC3 recruitment to *S. flexneri*-containing phagosomes, thereby inducing autophagy [128]. However, depletion of ATG5 does not inhibit TECPR1 recruitment to bacteria-containing phagosomes [127]. Instead, depletion of sphingomyelin by expression of neutral sphingomyelinase 2 (nSMase 2) impairs TECPR1 recruitment, suggesting that TECPR1 is recruited to sphingomyelin exposed on damaged membranes. Although depletion of TECPR1 alone does not inhibit LC3 localization to *Salmonella*-containing vacuoles (SCVs), simultaneous depletion of ATG16L and TECPR1 contributes to LC3 recruitment to SCVs. These findings indicate that while TECPR1 contributes to LC3 recruitment and autophagy, the ATG16L-dependent pathway is the primary route for LC3 recruitment in mouse embryonic fibroblast (MEF) cells [127]. TECPR1 recruitment precedes that of Gal-8; however, depletion of TECPR1 does not affect Gal-8 localization, suggesting that Gal-8 is recruited to SCVs independently of TECPR1[127].

In addition to endogenous TECPR1 and earthworm toxin lysenin, sphingomyelin can also be detected by equinatoxin II (EqtII), a PFT isolated from *Actinia equina* [129], and EqtII-GFP detects organelles after permeabilization [130,131]. Interestingly, Lysenin and EqtII do not colocalize completely [131]. In the presence of glycolipids that are miscible with sphingomyelin, the binding of lysenin to SM was decreased [132], indicating that lysenin binds to highly clustered sphingomyelin but not to sphingomyelin mixed with glycolipids. Lysenin stained large puncta in the plasma membrane as well as sphingomyelin in late endosomes, but not in the Golgi apparatus, early endosomes, or recycling endosomes [131]. In contrast, EqtII detects sphingomyelin in smaller puncta in the plasma membrane as well as in late endosomes and recycling endosomes [131]. EqtII can bind to sphingomyelin dispersed in glycolipids, thus EqtII can detect more sphingomyelin in diverse organelles. EqtSM is the modified EqtII domain without signal peptide and can be expressed in the cytosol to detect cytosol-exposed sphingomyelin [133].

2.3.2. Sphingomyelin Exposure in “Sterile” Membrane Damage

Sphingomyelin exposure has been detected in damaged endosomes or lysosomes induced by osmotic shock or LLOMe within 5-10 min, using Lysenin or EqtSM [127,133]. Sphingomyelin exposure is also rapidly observed at is the plasma membrane following laser-induced injury, occurring within 5 sec [133]. EqtSM is recruited to damaged plasma membrane upon treatment with streptolysin O (SLO), a PFT from *Streptococcus pyogenes,* as well as to damaged lysosomes following LLOMe or GPN *treatment. Chelation of* Ca^{2+} *by EGTA or BAPTA-AM significantly reduced Eqt-SM recruitment to both damaged plasma membrane and lysosomes [133]. Depletion of TMEM16F, a calcium-activated phospholipid scramblase localized in the plasma membrane, abolished EqtSM recruitment to SLO-damaged plasma membrane and to ionomycin-induced* Ca^{2+} *flux sites at the plasma membrane, but did not affect EqtSM recruitment to LLOMe-damaged lysosomes. These findings indicate that TMEM16F mediates sphingomyelin exposure at the plasma membrane but is dispensable for lysosomal sphingomyelin exposure. Notably, because BAPTA-AM also suppressed EqtSM recruitment to LLOMe-damaged lysosomes, an alternative* Ca^{2+} *-dependent scramblase is likely involved in lysosomal membranes. Depletion of ESCRT components, including CHMP3, ALIX, and TSG101, did not inhibit EqtSM recruitment, indicating that* sphingomyelin *exposure occurs independently of ESCRT-mediated membrane repair* [133]*. However, cell survival following LLOMe treatment was reduced upon depletion of either sphingomyelin synthase or ALIX and TSG101. These results suggest that sphingomyelin-dependent membrane responses contribute to cell*

 doi:10.20944/preprints202509.2282.v2

survival through a pathway that is functionally distinct from, yet complementary to the ESCRT repair machinery [133].

2.4. Stress Granules

Lysosomal damage induces stress granule formation. Various stress granule components were found to be associated with lysosomes via lysosomal immunoprecipitation after LLOMe treatment [134]. G3BP1 is a core protein to drive stress granule assembly by interacting with cytosolic RNAs by liquid–liquid phase separation [135,136]. Interestingly, stress granule formation occurs in a different location than lysosomes. After their formation, stress granules associate with damaged lysosomes [134,137]. The stress granule protein, NUFIP2, supports the inactivation of mTOR upon lysosomal damage to induce autophagy. Depletion of NUFIP2 keeps mTOR active, resulting in maintained autophagy pathway inactive [134]. G3BP1 forms condensates in damaged artificial vesicles with poly(A)-RNA at low pH, implicating the mixing of lower osmolarity and low pH solution with RNA leading to condensation of G3BP1[137]. Depletion of G3BP1 and 2 increased replication of *M. tuberculosis*, suggesting that stress granule plugs restrict bacterial proliferation in host cells [137]. Stress granule formation is triggered by various stresses, leading to phosphorylation of eIF2α and inhibiting global translation, thus resulting in the accumulation of untranslated mRNAs [138]. A search for proteins binding to eIF2α under LLOMe treatment identified PKR and its activator PACT as upstream kinases upon lysosomal damage to eIF2α [139]. ALIX binds to PKR and PACT under LLOMe treatment, and depletion of Ca^{2+} or ALIX inhibits phosphorylation of eIF2α, suggesting that Ca^{2+} leakage and recruitment of ALIX/ALG2 lead to phosphorylation of eIF2α for stress granule formation. The stress granule protein, NUFIP2, binds to Gal-8; however, depletion of Gal-8 does not inhibit stress granule formation [134]. On the other hand, depletion of Gal-3 increases stress granule formation, indicating that the Gal-3 pathway contributes to the repair of lysosomal damage [139]. Stress granule formation can be induced by various pathogenic lysosomal damage, including *M. tuberculosis*, Adenovirus infection, SARS-Cov-2 ORF3a protein, malarial pigment hemozoin, silica crystals, and Tau aggregates [134,139], indicating the importance of stress granule formation in attenuating the effects of lysosomal damage.

2.5. Rabaptin-5 and Rabs in Inducing Autophagy

Rabaptin-5 is a protein that binds to the small GTPases Rab5 and Rab4 and localizes to early and recycling endosomes [140–143]. Early and recycling endosomes can be damaged by chloroquine and monensin treatment [29]. Chloroquine accumulates in acidic compartments, where it acts as a weak base, absorbs protons, and induces swelling of endosomes or lysosomes through osmotic water influx. Monensin is an ionophore that perturbs Na^+ / H^+ exchange, thereby preventing acidification, primarily in recycling endosomes and the Golgi apparatus. Although Rabaptin-5 is a cytosolic protein and partially localizes in early and recycling endosomes under basal conditions [143], chloroquine treatment induces increased recruitment of Rabaptin-5 to endosomes within 15 min [29]. Chloroquine also promotes the recruitment of Gal-3, Gal-8, ubiquitin, FIP200, ATG16L and LC3 to endosomes within 15-30 min. Rabaptin-5 binds to the autophagy initiating factors FIP200 and ATG16L, and depletion of Rabaptin-5 inhibited LC3 recruitment to early or recycling endosomes [29], suggesting Rabaptin-5 promotes autophagy of damaged endosomes. In addition, depletion of Rabaptin-5 increases the survival of *Salmonella* internalized in HeLa and HEK293A cells, suggesting Rabaptin-5 contributes to the restriction of *Salmonella* survival.

Recently, many Rab GTPases have been shown to interact with autophagy receptors, predominantly NDP52 but also other receptors, thereby promoting autophagic degradation of the Rab proteins themselves [144]. Degradation of Rab proteins and their interaction with autophagy receptors depend on GTP-bound state of Rabs, suggesting that membrane-associated Rabs are selectively targeted for autophagy. Multiple Rabs (Rab1,2,5,8,14,18,19,21,27,28,34) are translocated to damaged mitochondria, and mutant of NDP52 that lacks Rab-binding domains impaired mitophagy. Rab2 and Rab18 are also recruited to lipid droplets, and depletion of Rab2 inhibited lipophagy.

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Salmonella-containing vacuoles (SCVs) recruit Rab9 and Rab14. While depletion of autophagy receptors does not impair recruitment of Rab9 and Rab14 to SCVs, mutation of the prenylation site of Rab9 and Rab14 disrupts their recruitment to SCVs, suggesting that translocation of Rabs to damaged organelles occurs upstream of NDP52-mediated recognition [144]. Together, these findings raise the possibility that Rabs function as upstream signals linking damaged organelles to selective autophagy pathways.

2.6. Membrane Contact Sites in Membrane Repair

2.6.1. Phosphatidylinositol-4 Phosphate (PI4P) in Lysosomal Damage

Phosphatidylinositol-4 phosphate (PI4P) localizes in the Golgi, endosomes/lysosomes and the plasma membrane [145]. Proteomic and lipidomic analyses of damaged lysosomes have revealed that PI4P is generated upon lysosomal injury [146,147]. PI4P can be visualized using PI4P-binding probes such as the pleckstrin homology (PH) domain of oxysterol-binding protein (OSBP-PH) and SidM(P4M) [148,149]. SidM is recruited to damaged lysosomes 5-10 min after LLOMe [146], and OSBP-PH is also recruited to damaged lysosomes within 30 min [147].

PI4P serves as a binding platform for lipid transfer proteins, including OSBP and OSBP-related proteins (ORPs) [145,150,151]. ORPs frequently localize to membrane contact sites in a PI4P-dependent manner at post-Golgi organelles, where they mediate the transfer of sterols or phosphatidylserine (PS) between post-Golgi organelles and the ER. Localization of OSBP-PH to the Golgi depends on both PI4P and Arf1, a small GTP-binding protein essential for vesicle budding at the Golgi[152]. Treatment of Brefeldin A (BFA), which inhibits the guanine nucleotide exchange factor required for Arf1 activation [153–155], disrupts Golgi localization of OSBP-PH [148], indicating that Golgi targeting of OSBP-PH requires both PI4P and Arf1. Notably, recruitment of OSBP-PH to damaged lysosomes after LLOMe treatment is independent of Arf proteins, as BFA treatment does not inhibit OSBP-PH recruitment [147].

PI4P production at damaged lysosomes requires phosphatidylinositol 4-kinase type IIα, PI4KIIA, since PI4KIIA depletion abolishes OSBP-PH recruitment to damaged lysosomes [147]. PI4KIIA itself is recruited to damaged lysosomes about 10 min after LLOMe treatment [147]. Importantly, activation of the lysosomal Ca^{2+} channel TRPML1 by its agonist ML-SA1 is sufficient to trigger PI4KIIA recruitment, suggesting that Ca^{2+} leakage is a key signal for recruitment of PI4KIIA to damaged lysosomes [147]. Interestingly, PI4KIIA recruitment is independent of ESCRT machinery. Depletion of ALIX and TSG101 does not affect PI4P production in damaged lysosomes, and conversely, depletion of PI4KIIA does not impair the recruitment of ALIX, CHMP4B, and Gal-3 to damaged lysosomes [146].

Within 10 min after LLOMe treatment, ORP1L, ORP9, 10, 11, and OSBP are recruited to damaged lysosomes [146,147]. ORP9, OSBP, and ORP1L contain an FFAT motif that binds to VAP-A and VAP-B localized in the ER and form membrane contact sites [145,151]. LLOMe treatment extensively increased ER–lysosome membrane contact sites [146,147]. Depletion of VAP-A/B attenuates the formation of ER–lysosome membrane contact sites [146]. Similarly, ORP9, 10, 11, and OSBP quadruple knockout (QKO) diminished VAP-A clustering around damaged lysosomes [147], suggesting that VAP-A/B and ORP form membrane contact sites in lysosomes upon lysosomal damage. ORP9 and 11 transfer phosphatidylserine (PS) to PI4P liposomes in vitro, and LLOMe treatment increased PS in lysosomes [147]. PS accumulation is rescued by any of two ORPs, ORP9/10, 9/11, or 10/11, and reduced Gal-3 recruitment to damaged lysosomes, implicating that PS transfer to damaged lysosomes plays a role in the repair of lysosomes.

Cholesterol also plays a role in repairing damaged lysosomes. OSBP transfers cholesterol, and depletion of OSBP decreased the survival of cells after LLOMe treatment. Accumulation of cholesterol in lysosomes decreases Gal-3 recruitment to damaged lysosomes, indicating that the presence of cholesterol protects lysosomes from damage as well as PS [146].

 doi:10.20944/preprints202509.2282.v2

PI4P also accumulates in lysosomes when the production of $PI(3,5)P_2$ is inhibited [156]. The inhibitors of the PIKfyve complex, which converts PI(3)P to $PI(3,5)P_2$, promote the translocation of PI4KIIA from the TGN to lysosomes. While LLOMe treatment induces Gal-3 recruitment to damaged lysosomes, pretreatment with PIKfyve inhibitors attenuates Gal-3 recruitment. These findings suggest that increased levels of PI4P and PI4KIIA in lysosomes upon PIKfyve inhibition mitigate lysosomal damage [156]. Although Apilimod, a PIKfyve inhibitor, preserves lysosomal acidity and thus limits overt lysosomal membrane damage, it enhances recruitment of ORP1L to membrane contact sites between the ER, lysosomes and mitochondria, suggesting membrane repair processes are activated [156]. Apilimod has also emerged as a potential therapeutic strategy for neurodegenerative diseases by promoting the secretion of neurotoxic proteins [157]. Apilimod treatment increases the secretion of aggregation-prone proteins, including phosphorylated TDP-43. This secretion is inhibited by GW4869, an inhibitor of neutral sphingomyelinase 2, as well as by suppression of genes involved in exosome exocytosis [158] or amphisome-mediated secretion [157,159]. Together, these findings suggest that mild lysosomal stress induced by inhibition of PI(3)P-to-$PI(3,5)P_2$ conversion enhances another endosome/lysosome repair pathway, namely, the exocytic disposal of contents of MVBs and lysosomes. Interestingly, CD63-positive MVBs are also positive for PI4P (See also section 2.7.3), and inhibition of PI4KIIA resulted in inhibition of recruitment of Exocyst complex that mediates the fusion of secretory vesicles to the plasma membrane [160]. Future studies will reveal the role of PI4P in secretory MVBs.

2.6.2. ATG2-ATG9 in Membrane Damage

ATG2, originally discovered as a required component for autophagy in yeast [37,38,161,162], possesses a cavity to bind to lipids and has lipid transfer activity [163–165]. Similarly, the human homologs ATG2A and ATG2B have PS transfer activity [165,166]. ATG2A localizes in lysosomes after LLOMe treatment in 5-10 min [147]. Expression of mutant ATG2A that lost lipid transfer activity of PS to PI4P-positive liposomes in vitro, maintains Gal-3 puncta longer than WT ATG2A expression, suggesting that PS transfer of ATG2A to lysosomes helps to repair damaged lysosomes [147].

ATG9, another essential protein for autophagy [167,168], helps to repair the damaged plasma membrane [103] and lysosomes [169]. ATG9 is a multi-membrane spanning protein and cycles between TGN and late and recycling endosomes [170,171]. When the autophagy pathway is activated, ATG9 relocalizes to precursors of the autophagosome [171,172]. ATG9 forms a homotrimer complex [173] and has lipid scramblase activity [174]. ATG9 binds to ATG2 and is thought to supply phospholipids to the autophagosome for autophagosome membrane expansion [174,175]. ATG9 binds to many of the vesicle budding machineries. ATG9 binds to clathrin adaptors, AP-2 [176,177], AP-4 [178,179], AP-1 [180], and AP-3 [169], as well as retromer complex [176]. When AP-2 or AP-4 are depleted, ATG9 remains in the recycling endosomes or TGN even under starvation conditions, and the autophagy pathway is inhibited, suggesting proper transport of ATG9 is required for autophagosome formation [176–178].

When the plasma membrane was damaged by digitonin, SLO, or glass-beads injury for 1-10 min, ATG9 can be recruited to the plasma membrane [103]. Plasma membrane permeabilization was exacerbated in ATG9-depleted cells indicating that ATG9 protects plasma membrane from damage [103]. ATG9 is recruited to the injured plasma membrane in a manner dependent on IQGAP1, which responds to Ca^{2+}, and depletion of Ca^{2+} outside of cells inhibits ATG9 recruitment to the injured plasma membrane [103]. IQGAP1 interacts with ATG9 and CHMP2A, ESCRT-III protein, and double knockout of ATG9 and CHMP2B does not exacerbate PM permeabilization compared to single knockout of ATG9 or CHMP2B, which indicates that ATG9 and CHMP2B function in the same pathway for plasma membrane repair.

ATG9 has also been reported to play a role in lysosomal repair as well [169]. In LLOMe-treated cells, ATG9 is recruited to damaged lysosomes within 15-45 min [169]. Depletion of cellular Ca^{2+} inhibited ATG9 recruitment, indicating that Ca^{2+} promotes ATG9 recruitment [169]. ATG9 vesicles contain ARFIP2/Arfaptin 2, an Arf-GTP binding protein [152], and bind to PI4P and PI4PK2A

 doi:10.20944/preprints202509.2282.v2

[169,181]. Depletion of ARFIP2 increases ATG9, PI4KIIA, and PI4P localization in damaged lysosomes, and lysosomes are repaired faster [169]. ARFIP2 forms a complex with AP-3 and, when AP-3 is depleted, ATG9 increases in damaged lysosomes, implicating that AP-3 helps to retrieve ATG9 from the endolysosomal compartment to the Golgi and attenuates lysosomal repair [169]. ARFIP2 binds to ORP9 and interferes with the PS lipid transfer activity of ORP9/11 in vitro [169]. Depletion of ARFIP2 restricts *Mtb* and *Salmonella* infection, demonstrating that ARFIP2 inhibits lysosomal repair under normal conditions, while depletion of ARFIP2 promotes lysosomal repair [169]. These findings indicate that the transfer of PS and cholesterol at the expense to PI4P contributes to membrane repair, mediated by ORP9-11, OSBP, or ATG2-ATG9 complex. In contrast, clathrin adaptors and ARFIP2 restrict the repair process by regulating ATG9 trafficking.

ATG9 vesicles transport PI4KIIA from the TGN to lysosomes [156]. Inhibition of the PIKfyve complex, which converts PI(3)P to PI(3,5)P_2, increases the levels of PI4P and PI4KIIA in lysosomes. In contrast, depleting ATG9 prevents PI4KIIA localization to lysosomes, indicating that ATG9 is required for PI4KIIA transport from the TGN to lysosomes.

2.7. Annexins

2.7.1. Annexins in Plasma Membrane Repair

Annexins are a family of Ca^{2+}-dependent phospholipid-binding proteins [182,183]. Annexins bind to negatively charged phospholipids, including PS, PI, and PI(4,5) P2, in a manner dependent on Ca^{2+} [184–190]. Among them, PS is most characterized and largely used for Ca^{2+}-dependent binding of Annexins to lipids. In addition, Annexin A2 binds to cholesterol independent of Ca^{2+}[191].

Annexins A1 and A2 associate with Dysferlin, whose mutation causes dysferlinopathy [192]. Dysferlin-deficient myotubules lose the ability to reseal the damaged plasma membrane [192]. Lack of Annexin A2 impairs repair of injury in a Cholesterol-dependent manner in muscle cells [193]. Annexins also affect plasma membrane repair in non-muscle cells. As shown via the scraping procedure in HeLa and kidney epithelial cells, Annexin A1 deficient for Ca^{2+} binding loses the ability to reseal plasma membrane injury [194]. Annexins A4, 5, 6, and 7 are also involved in plasma membrane repair [102,195,196].

Annexins A1, 2, and 6 translocate to the wound area in a wave-like manner and form a ring-like structure around the wound within 2-30 sec [197]. Annexins A1, 2, 5, and 6 localize in the repair cap as well after injury in 50 sec, dependent on Ca^{2+} and actin [198]. Annexin A5 forms a trimer to form a 2D array via self-assembly on PS-exposed membranes [196]. An Annexin A5 mutant that disrupt its trimer structure inhibited membrane repair; however, membrane binding was not inhibited [196], suggesting that self-assembly of Annexin A5 is required for membrane repair but not membrane binding.

Annexins A4 and A6 can regulate membrane curvature [195]. Annexin A4 binds to the edge of the membrane and rolls up the membrane. In contrast, Annexin A6 can constrict the planar membrane. As determined via live imaging, Annexin A6 was recruited to the damaged area before Annexin A4 and localized as a ring-like structure around the wound. It has been proposed that Annexin A6 binds to the edge and Annexin A4 rolls up the hole of the edge, following which Annexin A6 constricts to close the membrane. This process completed within 15 sec in a mammary carcinoma cell line, MCF7 cells [195].

Annexin A7 also plays a role in plasma membrane repair [102]. Annexin A7 binds to ALG2 and recruits ALG2 to the plasma membrane wound within 100 sec. ALG2 is accumulated at the edge of the membrane in an Annexin A7-dependent manner. A mutant of Annexin A7 that cannot be recruited to the lesion of the plasma membrane inhibited ALG2 recruitment. CHMP4B localized at the repair site, and shed vesicles were observed from the plasma membrane within 2-15 min. The ESCRT complex induces shedding of the plasma membrane as microvesicles/ectosomes for membrane repair. The findings of these studies indicate that Annexins function as first-line

 doi:10.20944/preprints202509.2282.v2

machinery for plasma membrane damage detection and repair. ESCRT-mediated membrane repair follows the repair initiated by Annexin A7.

2.7.2. Annexins in Endolysosomal Repair

Annexin A2 is recruited to damaged multi-vesicular bodies (MVBs) as well as lysosomes in dendritic cells (DCs) [26]. Ultra-high molecular weight polyethylene is released from joint replacement as wear debris, and induces inflammation when endocytosed by macrophages and DCs [199]. Wear debris comprising nanometer- to micrometer-sized particles are incorporated into MVBs and release lysosomal enzymes such as Cathepsin S and B [26,199], suggesting the internalization of wear debris damages MVBs and lysosomes. Depletion of Annexin A2 results in increases in the release of Cathepsin B and IL-1b [26]; therefore, Annexin A2 would repair damaged MVBs and lysosomes.

Annexins A1 and 2 play critical roles in lysosomal repair following LLOMe treatment in human osteosarcoma U2OS cells [200]. Although multiple Annexins are recruited to damaged lysosomes within 10-30 min, only Annexins A1 and A2 exhibit lysosomal repair activity. Notably, CHMP4B is recruited to damaged lysosomes even in cells depleted of Annexin A1 and A2, and conversely, Annexin A1 and A2 are recruited to damaged lysosomes in ALIX and TSG101-depleted cells. These findings indicate that the Annexin A1 and A2-mediated repair pathway operates independently of ESCRT machinery, in contrast to plasma membrane repair (see section 2-7-1). Recruitment of Annexin A1 and A2 is impaired by Ca^{2+} chelation, demonstrating that their recruitment to damaged lysosomes is also calcium dependent [200]. Furthermore, Annexins A1 and 2 are preferentially recruited to lysosomes sustaining larger injuries that permit the release 10-KDa dextran, whereas ESCRT components can be recruited to even to lysosomes with smaller membrane injuries.

Annexin A7 has also been reported to be important for lysosomal repair in MCF7 and HeLa cells [201]. Annexin A7 can be recruited to damaged lysosomes by LLOMe within 15 min, and depletion of Annexin A7 exacerbates Gal-3 recruitment to damaged lysosomes. Although Annexin A7 is known to bind to ALG2 [102], CHMP4B recruitment to damaged lysosomes is not affected in Annexin A7-depleted cells [201]. In addition, Annexin A7 depletion does not affect lysophagy or alter lipid composition, which is regulated by the PI4P pathway [146,147]. Thus, Annexin A7 facilitates lysosomal repair through other mechanisms.

We also note that the timing of Annexin recruitment to damaged lysosomes occur more slowly (10-30 min) than recruitment to damaged plasma membrane (2-50 sec; see Table 1). Together, these findings suggest that Annexins contribute to lysosomal repair through mechanisms distinct from those operating during plasma membrane repair.

2.7.3. Annexins for Plasma Membrane Repair by Fusion of Secretory Lysosome/MVB

Upon plasma membrane damage, it is proposed that secretory lysosomes fuse with the damaged area of the plasma membrane in a Ca^{2+}-dependent manner to repair plasma membrane damage [202]. Exosomes are endosome-derived extracellular vesicles, which are secreted by a wide variety of cells, including cancer cells and platelets [158]. When multivesicular bodies (MVBs) fuse with the plasma membrane, their internal luminal vesicles (ILVs) are released as exosomes [203–205]. The tetraspanin protein CD63 localizes in ILVs of MVBs and is secreted on exosomes [206]. Exosomal release can be induced by Ca^{2+} in HCT116, a human colon cancer cell line, and plasma membrane damage by streptolysin O (SLO) also stimulates exosome secretion [207].

Annexins A2 and 6 have been identified as proteins recruited to CD63-positive MVBs in a Ca^{2+} dependent manner [207]. Depletion of Annexin A6 inhibited MVB fusion and release of exosomes stimulated by Ca^{2+} ionophore [207]. Moreover, an antibody against Annexin A6 prevents SLO-stimulated exosome release, indicating that Annexin A6 mediates the fusion of MVBs to the damaged plasma membrane. The depletion of Annexin A2 also inhibited CD63-posivite exosomal release in hepatocellular carcinoma [208]. It has been known that Annexin A2 has endosome fusion activity [209], and plays a role in exocytosis of secretory granules in chromaffin cells [210]. Annexin A2 is

 doi:10.20944/preprints202509.2282.v2

translocated to the plasma membrane after exocytosis, in a manner dependent on lipid rafts. It has been proposed that Annexin A2 forms a lipid raft domains in the plasma membrane in a Ca^{2+}-dependent manner and mediates the fusion of secretory granules with the plasma membrane [210]. Similar mechanisms may also be involved in the fusion of CD63-positive MVBs to the plasma membrane.

These findings suggest that Annexin A2 and A6 play roles in the fusion of CD63-positive MVBs with the damaged plasma membrane to facilitate plasma membrane repair as well as releasing exosomes.

4. Conclusion

Studies of bacterial infection and damage to organelle membranes have significantly advanced our understanding of how cells detect organelle membrane damage. Some molecules are shared between the detection of plasma membrane and lysosomal membrane damage, whereas others are specific to each organelle. The key molecules are summarized in Table 1, and Figure 1 illustrates their association with each organelle.

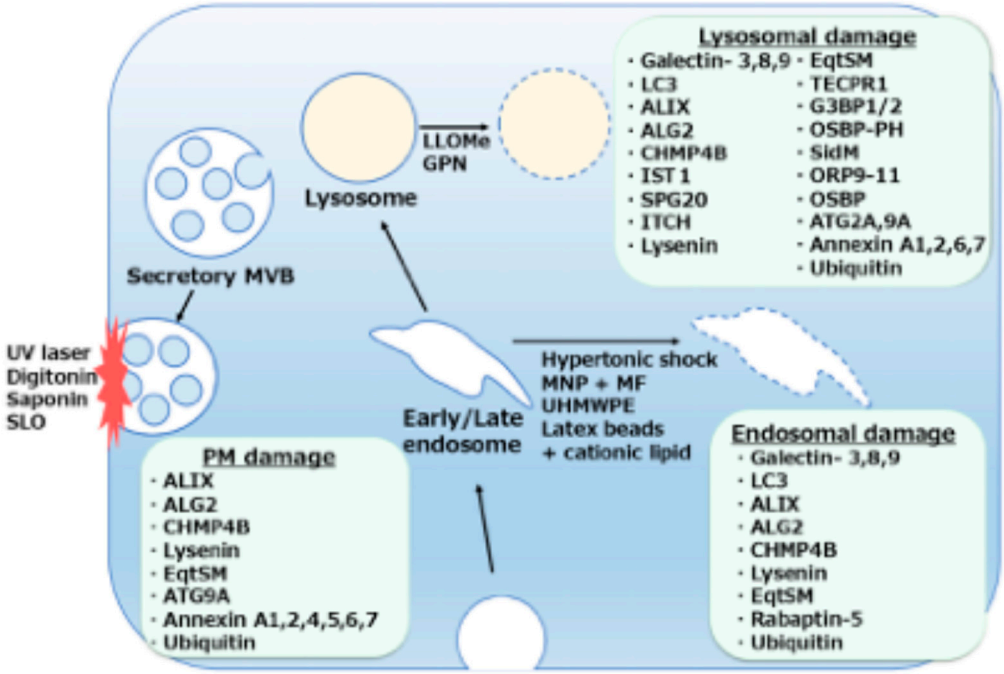

Figure 1. Schematic model of molecules recruited to damaged organelles and artificial damaging methods. This figure illustrates the key factors involved in membrane damage detection in different organelles, as described in this review. Various artificial methods used to induce organelle damage are also indicated. Abbreviations not used in the main text: NP, nanoparticle; MNP, magnetic nanoparticle; MF, magnetic field; UHMWPE, Ultra-high molecular weight polyethylene.

Table 1. Molecules recruited to damaged organelles. This table summarizes the molecules discussed in this review. Binding sites are listed only for interactions that occur upon membrane damage and do not include sites or proteins to which the listed molecules bind under non-damaged conditions (e.g., OSBP binding to Arf1, or Rabaptin-5 binding to Rab4 and Rab5). The "Timing" column indicates when each molecule is recruited to damaged organelles following damage-inducing treatments. Earlier recruitment events may be identified in future studies using improved live-cell imaging or advanced microscopy techniques.

Molecule	Binding sites upon membrane damage	Recruitment upon membrane damage	Timing	reference
Galectin-3	luminal glycosylation	lysosomes, endosomes	10-30min	[39,40,43,44]
Galectin-8	luminal glycosylation	lysosomes, endosomes	30-60min	[59]

 doi:10.20944/preprints202509.2282.v2

Galectin-9	luminal glycosylation	lysosomes, endosomes	5-10min	[61–63]
LC3		lysosomes, endosomes, BCV[a]	30min	[39,40,43,44,59]
Ubiquitin		Lysosomes, BCV, endosomes plasma membrane	30-60min 6min	[29,48,51,89]
RNF213		BCV	2hr	[50]
Parkin		BCV	4hr	[52]
Smurf1		BCV	15hr	[53]
LRSAM1		BCV	45min	[54]
UBE2QL1		Lysosomes	30min	[48]
ALIX	ALG2 binding under Ca^{2+}, Gal-3 binding	Lysosomes plasma membrane	10min 30sec	[27,57,89,90,104,105]
ALG2	Ca^{2+}	plasma membrane	30sec	[90,100,102]
CHMP4B	ALIX/TSG101	lysosomes, endosomes plasma membrane	10min 10-60sec	[27,57,89,90,104,105]
IST1	ALG2 binding under Ca^{2+}	Lysosomes	60sec	[111,112]
SPG20	Peroxidized lipids, IST1	Lysosomes	5min	[111]
ITCH	SPG20 binding	Lysosomes	15min	[111]
Lysenin	Sphingomyelin exposure to cytosol	lysosomes, endosomes BCV	10min 30min	[125,126]
EqtSM	Sphingomyelin exposure to cytosol	lysosomes plasma membrane	5min 5sec	[131,133]
TECPR1	Sphingomyelin exposure to cytosol	BCV, lysosomes	5-30min	[127,128]
G3BP1/2		Stress granules partially overlapped with damaged lysosomes	2-30min	[134,137,139]
Rabaptin-5		Endosomes	30min	[29]
PI4KIIA	Ca^{2+} leakage	Lysosomes	10min	[147]
SidM	PI4P	Lysosomes	5-10min	[146,169]
ORP9-11	PI4P	Lysosomes	10min	[147]
OSBP-PH	PI4P	Lysosomes	30min	[146,147]
ATG2A		Lysosomes	10 min	[147]
ATG9A	Ca^{2+}	Lysosomes plasma membrane	15-45min 1-10min	[103,169]
Annexin A1,2,4,5,6,7 Annexin A1,2,7	Ca^{2+}, negative-charged lipids	plasma membrane MVBs, lysosomes,	2-50 sec 10-30min	[26,102,195–198,200,201,207]

[a] BCV; bacteria-containing vacuoles.

In general, early recruitment events are largely dependent on calcium release, which triggers the recruitment of ALG2/ALIX together with the ESCRT complex, ATG9 vesicles, and annexins. These events occur at both the plasma membrane and damaged lysosomes, although not all molecules are universally involved in both contexts. Notably, the recruitment of sensor molecules occurs considerably faster following plasma membrane damage than after lysosomal membrane damage, suggesting that plasma membrane damage requires more rapid repair than lysosomal damage.

Although we attempted to identify molecules involved in sensing endosomal membrane damage, we also noted that membrane damage in one organelle may indirectly affect the function of other organelles. For example, when the plasma membrane is damaged, secretory MVBs/lysosomes may fuse with the plasma membrane, suggesting that endosomal/lysosomal molecular machineries, such as Annexin A6 are altered in response to plasma membrane damage. Similarly, when lysosomes are damaged, PI4KIIA is recruited from the TGN, suggesting that TGN-associated machineries are also affected by lysosomal damage. Therefore, alterations in molecules associated with a particular organelle may include indirect effects induced by membrane damage in other organelles. These processes remain incompletely understood and will require further investigation in future studies.

 doi:10.20944/preprints202509.2282.v2

Membrane damage responses are increasingly recognized as integral components of broader cellular stress responses, with implications extending to autophagy, inflammation, and unexpectedly, exosome biology. Continued discoveries in this field are expected to expand the list of key factors involved, thereby contributing to the development of DDS strategies and advancing a wide range of biomedical research.

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