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Biochemical and Biophysical Research Communications 606 (2022) 156–162

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Clathrin-mediated trafficking of phospholipid flippases is required for local plasma membrane/cell wall damage repair in budding yeast

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ARTICLE INFO

Article history:
Received 23 February 2022
Received in revised form
9 March 2022
Accepted 23 March 2022
Available online 24 March 2022

Keywords:
Plasma membrane repair
Clathrin
Phospholipid flippase
Exocyst
Exo70
Budding yeast

ABSTRACT

Plasma membrane damage and repair frequently happen in cells. A critical process underlying plasma membrane repair is to redirect repair factors, such as protein kinase C and the exocyst complex, from the polarized site to the damage site. However, the mechanism underlying the repair factor delivery to the damage site remains unknown. Here, we demonstrate that clathrin-mediated trafficking of repair factors is involved in plasma membrane/cell wall repair in budding yeast. Using laser-induced plasma membrane/cell wall damage assay, we identified phospholipid flippases, Lem3-Dnf1/Dnf2 and Cdc50-Drs2, as essential clathrin cargos for plasma membrane/cell wall repair. We found that flippase impairment significantly compromised the recruitment of exocyst Exo70 to the damage site. In contrast, the recruitment of protein kinase C (Pkc1) was only mildly compromised. Taken together, clathrin-mediated trafficking of the phospholipid flippases is critical for the recruitment of exocyst to the damage site. Mechanisms to redirect exocyst via the clathrin and flippase-mediated pathways may be a general feature of effective plasma membrane repair in polarized cells.

1. Introduction

Cells frequently experience physiological and pathological plasma membrane damages such as cardiac muscle injuries and pore formation by bacterial toxins [1,2]. Therefore, cells are equipped with evolutionally conserved membrane repair mechanisms. Plasma membrane repair is an energy-dependent process, which requires physiological concentration of Ca^{2+} [1,3]. Ca^{2+} influx after plasma membrane damage triggers rapid membrane repair processes including exocytosis and endocytosis [3–5]. Deficits in plasma membrane damage response are associated with multiple diseases including limb-girdle muscular dystrophy [6] and Scott syndrome [7,8].

It is beneficial to study complex and evolutionarily conserved mechanisms in genetically tractable model organisms including yeasts. Using budding yeast, we previously showed that laser-induced local plasma membrane/cell wall damage induces recruitment of repair factors such as protein kinase C (Pkc1), linear actin nucleator formin (Bnr1), type V myosin (Myo2), and exocyst (Exo70) to the damage site [9]. To identify the gene sets required for the plasma membrane/cell wall damage responses, we performed a genome-wide screening using budding yeast gene deletion libraries [10]. We identified 48 genes that are required for survival in the presence of local plasma membrane/cell wall damage [10]. These screening hits included the gene sets involved in protein trafficking, raising a possibility that protein trafficking is mechanistically involved in plasma membrane/cell wall damage responses.

In this study, we identified clathrin-mediated trafficking of phospholipid flippases as an important mechanism underlying plasma membrane/cell wall repair in budding yeast. We also revealed that the flippases contribute to the recruitment of repair factor Exo70 to the damage site. Our study demonstrates novel functions of clathrin-mediated trafficking and phospholipid flippases during plasma membrane/cell wall repair in budding yeast.

2. Materials and methods

2.1. Media and strains

Standard procedures were used for DNA manipulations, *E. coli*, and yeast genetic manipulations. Yeast transformations were

Abbreviations: GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; SDS, sodium dodecyl sulfate.

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https://doi.org/10.1016/j.bbrc.2022.03.129

performed by lithium acetate method. For gene deletion, PCR based procedure was used. The deletion of the expected locus was confirmed by colony PCR [11]. Yeast cells were cultured in yeast extract peptone dextrose (YPD) media unless otherwise indicated. SD-Trp media were used for laser damage assay. Yeast culture was performed at 25 °C unless otherwise indicated. The yeast strains and plasmids used in this study are listed in Tables S1 and S2. For Fig. 4, Fig. S3, Fig. S4, and Fig. S5, yeast culture was performed at 30 °C because *lem3*Δ*cdc50*Δ grew healthier at 30 °C than 25 °C.

2.2. Spot assay

The overnight yeast culture in YPD at 25 °C were diluted to OD_{600} 0.1 and 4-fold serial dilutions were spotted onto the indicated plates. The plates were incubated at indicated temperature for 3–4 days.

2.3. Viability assay

The log phase ($OD_{600} < 0.7$) yeast cultures in YPD at 25 °C were diluted to $OD_{600} = 0.001$ and 100 μl of the cultures were plated onto YPD plates. Subsequently, SDS was added (final concentration, 0.02%). The cultures were incubated for 2- or 2.5-h at 25 °C and 100 μl of the cultures were plated onto YPD plates. The plates were incubated at 25 °C for 3–4 days. The colony numbers were counted.

2.4. DAPI penetration assay

Yeast cells were grown overnight in YPD media at 25 °C ($OD_{600} < 0.7$). The cultures were diluted to $OD_{600} = 0.2–0.4$. The liquid media were switched to YPD +2 ng/ml DAPI (Control) and YPD + 0.02% SDS + 2 ng/ml DAPI (SDS+). The cultures were incubated at 25 °C for 30 min. The cells were fixed with 4% paraformaldehyde in YPD before observation under Celldiscoverer 7 (Zeiss).

2.5. Laser damage assay

Laser damage assays were performed as previously described [9] with modifications. Yeast cells were grown overnight in SD-Trp media at indicated temperature to early log phase (OD_{600} ~0.4). The cultures were refreshed and incubated for 3–8 h until OD_{600} reached to 0.1–0.3. Cells were spotted onto agarose containing media (SD-Trp + 2.2% agarose) on ConA (Nacalai) coated coverslips. A1R HD25 confocal microscopy (Nikon) was used to induce cell wall and plasma membrane damage and to monitor the fluorescence. 405 nm laser was used to induce damage. Signal quantification was performed using FIJI software [12]. We defined recruitment when the normalized signal at the damaged cortex was greater than three standard deviations above the background signal from a non-damaged cortex region for at least six consecutive time frames (20 s/frame).

3. Results

3.1. The genes involved in protein trafficking, SWA2, VPS16, and VMA21, are required for the survival after plasma membrane/cell wall damage

We previously identified 48 genes that are potentially involved in the plasma membrane/cell wall damage response in budding yeast [10]. In the screening, we identified the mutants sensitive to 0.02% sodium dodecyl sulfate (SDS) because this condition induces local plasma membrane and cell wall damage [10]. The genes involved in protein trafficking were enriched in the screening hits. To test the possibility that protein trafficking is critical for the plasma membrane/cell wall damage response in budding yeast, we picked nine screening hits whose gene functions are associated with protein trafficking and signal transduction. To distinguish whether the mutants died, or their cell cycle progression was arrested after plasma membrane/cell wall damage, we assessed cell viability after incubation with YPD +0.02% SDS for 2.5 h. We found that *swa2*Δ, *vps16*Δ, and *vma21*Δ cells lost their viability after SDS treatment (Fig. S1A), supporting the idea that *SWA2*, *VPS16*, and *VMA21* are involved in survival after plasma membrane/cell wall damage. To further test the possibility, we examined whether transient 0.02% SDS treatment (30min) leads to the penetration of a scarcely membrane permeable dye 4',6-diamidino-2-phenylindole (DAPI) into these mutants (Fig. S1B and C). Consistent with the viability assay, *swa2*Δ, *vps16*Δ, and *vma21*Δ cells showed higher DAPI penetration rate than WT (Fig. S1C). These results are consistent with our working hypothesis that *SWA2*, *VPS16* and *VMA21* are required for plasma membrane resealing.

3.2. Clathrin-mediated trafficking is involved in plasma membrane/cell wall repair

SWA2 encodes a yeast Hsp40 chaperone triggering disassembly of clathrin, which is essential for clathrin-mediated protein trafficking including endocytosis and sorting of proteins at trans-Golgi network (TGN) [13–16]. Therefore, we hypothesized that clathrin-mediated protein trafficking is involved in plasma membrane/cell wall repair. Although we did not identify clathrin mutants in our initial screen using the gene deletion libraries, newly constructed *clc1*Δ and *chc1*Δ mutants, where clathrin light chain (*CLC1*) or heavy chain (*CHC1*) were deleted, were sensitive to 0.02% SDS (Fig. 1A). Moreover, their viability after SDS treatment was significantly decreased (Fig. 1B); their DAPI penetration rates were significantly higher than that of WT (Fig. 1C and D). These results suggest that clathrin-mediated protein trafficking is required for plasma membrane/cell wall damage response upon SDS treatments.

3.3. Phospholipid flippases are the clathrin cargo required for the local plasma membrane/cell wall repair

To understand the roles of clathrin-mediated protein trafficking in plasma membrane/cell wall repair, we investigated the involvement of clathrin cargo proteins. There were several proteins, such as chitin synthase 3 (Chs3) [17] and vesicle membrane receptor protein (Snc1) [18], whose localization is dependent on clathrin-mediated trafficking. Among these proteins, we focused on two phospholipid flippases, Dnf1 and Drs2. Dnf1 and Drs2 are functionally associated with Sla1-dependent endocytosis [19]. In addition, TGN-localized Drs2 is recycled by clathrin adaptor AP-1 [20]. Phospholipid flippases translocate specific phospholipids from the outer leaflet to the inner leaflet so that lipid asymmetry is established and maintained [21]. Therefore, we hypothesized that these phospholipid flippases are recruited to the damage site and remodel the plasma membrane lipid asymmetry after damage. To test this hypothesis, we performed the laser damage assay to induce local plasma membrane/cell wall damage in yeast [9]. We found that Dnf1-GFP and Drs2-GFP locally accumulated at the laser damage site (Fig. 2A and B), indicating that Dnf1 and Drs2 are involved in the plasma membrane/cell wall repair. Dnf1 and Drs2-GFP accumulation at the damage site was significantly impaired in *clc1*Δ cells (Fig. 2A and B). These results suggest that clathrin-mediated protein trafficking mediates the recruitment of flippases to the damage site.

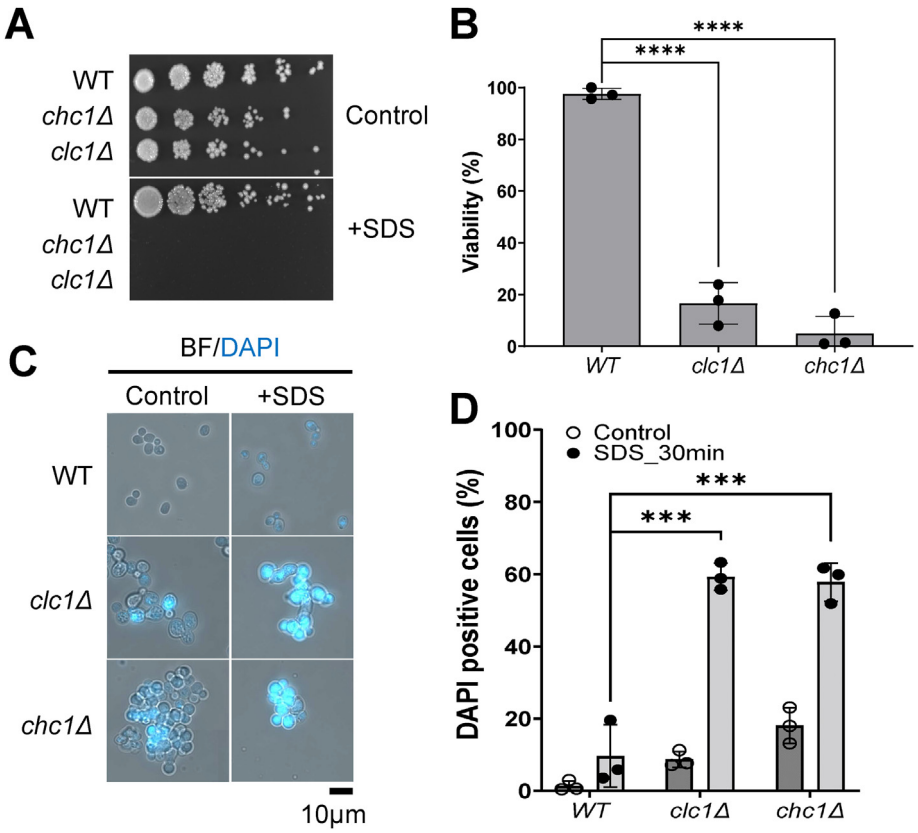

Fig. 1. Clathrin-mediated trafficking is involved in cell wall/plasma membrane repair after SDS treatment. (A) 4-fold serial dilutions were spotted onto YPD and YPD + 0.02% SDS plates, followed by incubation for 3 days at 25 °C. (B) The viability after 0.02% SDS treatment for 2 h was measured. Mean ± SD of 3 independent experiments are presented. One-way analysis of variance (ANOVA) test and the post hoc Bonferroni test were performed. *P*-values: ****p < 0.0001. (C) Yeast cells at 30 min after adding 2 ng/ml DAPI and 0.02% SDS (SDS+) or without SDS (Control) were observed. BF: Bright field. (D) DAPI positive cells were counted (>100 cells/sample). Mean ± SD of 3 independent experiments are presented. One-way analysis of variance (ANOVA) test and the post hoc Bonferroni test were performed. *P*-values: ***p < 0.001.

3.4. Dnf1 is recycled to the damage site via Sla1-dependent endocytosis

Clathrin-mediated trafficking is regulated by adaptor proteins such as AP-1, AP-2, GGA, and Sla1 (Fig. 3A) [22,23]. AP-1 mediates intra-Golgi recycling of TGN proteins [24,25]. GGA mediates the trafficking of Golgi to late endosomes [18]. AP-2 and Sla1 mediates endocytosis [23]. We found that AP-1 deleted mutant (*apl2*Δ), GGA deleted mutant (*gga1*Δ*gga2*Δ), and *sla1Δ* cells showed sensitivity to 0.02% SDS (Fig. 3B). These results indicate that AP-1, GGA-mediated trafficking, and Sla1-dependent endocytosis are involved in plasma membrane/cell wall repair. Therefore, we hypothesized that flippases require these adaptor proteins for their targeting to the damage site. To test this hypothesis, we performed the laser damage assay using adaptor deleted mutants (Fig. 3C, Fig. S2A). We found that, in *apl2*Δ cells and AP-2 deleted mutants (*apl1*Δ), Dnf1-GFP accumulation at the damage site was not significantly impaired (Fig. S2A–C). These results indicate that AP-1 and AP-2 are dispensable for the Dnf1 recruitment to the damage site. In control cells, eight out of 10 cells (80%) showed Dnf1-GFP accumulation. However, in *gga1*Δ*gga2*Δ cells, only six out of 14 cells (43%) showed Dnf1-GFP accumulation, although the kinetics of accumulation were comparable to that of controls (Fig. S2A and D). These results suggest that GGA adaptors may play a minor role in Dnf1 trafficking. Dnf1-GFP accumulation at the damage site was significantly impaired in *sla1*Δ cells (Fig. 3C and D), consistent with previous reports that Dnf1 was recycled via Sla1-dependent endocytosis under unperturbed growth conditions [19]. These results suggest that, after plasma membrane/cell wall damage, Dnf1 is recycled from bud to the damaged site via Sla1-dependent endocytosis.

3.5. Phospholipid flippases are required for recruiting plasma membrane repair factor Exo70 to the damage site

Budding yeast has five phospholipid flippases, Drs2, Dnf1, Dnf2, Dnf3, and Neo1. Neo1 is essential for cell viability; other flippases work redundantly in controlling membrane asymmetry [26,27]. Flippases form heterodimeric complexes with non-catalytic subunits Cdc50, Lem3, and Crf1. Dnf1 and Dnf2 form complexes with Lem3, while Drs2 and Dnf3 interact with Cdc50 and Crf1, respectively. Except for Neo1, these interactions are required for endoplasmic exit and proper localization of flippases [26]. Therefore, phenotypes of flippase mutants phenocopies mutants of their subunits.

To evaluate the roles of phospholipid flippases in plasma membrane repair, we tested SDS sensitivity of flippase mutants; *lem3*Δ, *cdc50*Δ, and *lem3*Δ*cdc50*Δ. We found that *lem3*Δ, *cdc50*Δ, and *lem3*Δ*cdc50*Δ cells showed sensitivity to 0.02% SDS. In contrast, only *lem3*Δ*cdc50*Δ showed sensitivity to lower concentrations of SDS (0.01% and 0.005%) (Fig. 4A). These results suggest that Lem3-

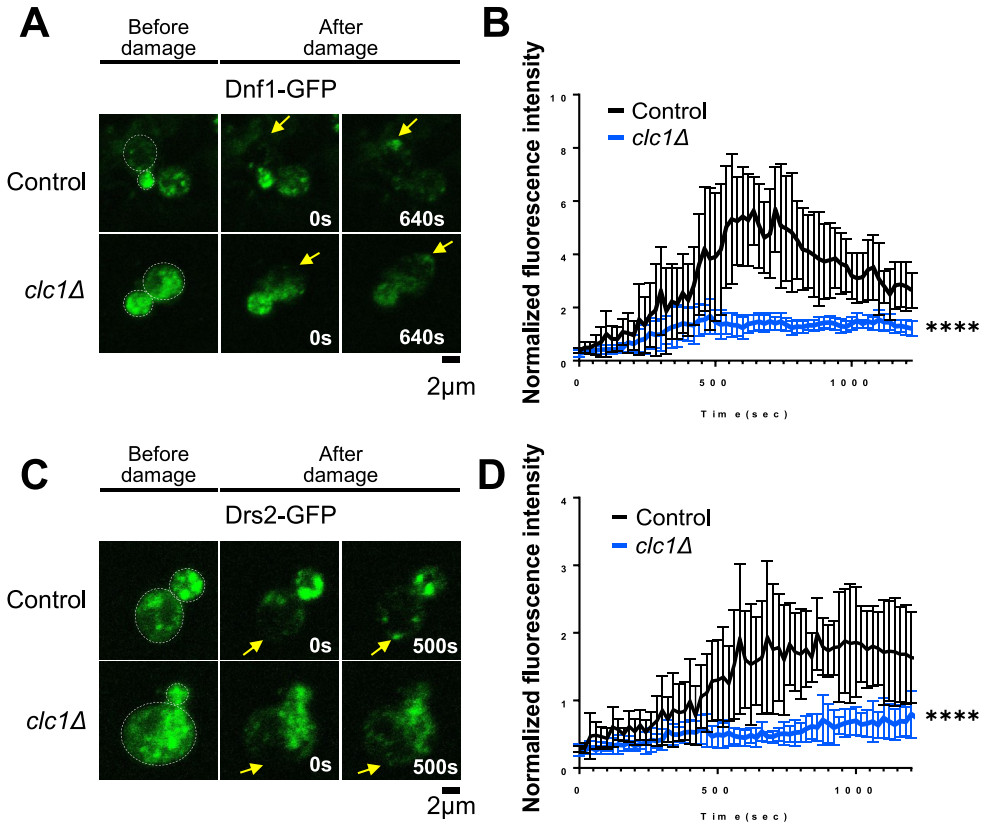

Fig. 2. Phospholipid flippases' accumulation at the laser damage site requires *CLC1*. (A) Dnf1-GFP and (C) Drs2-GFP in control and *clc1Δ* were monitored before and after 405 nm laser damage. Yellow arrows indicate laser damage sites. (B) and (D) show fluorescence signal intensity of Dnf1-GFP and Drs2-GFP at the damage site, respectively. Fluorescence signal intensity at the damage site was normalized by whole cell fluorescence intensity. *P*-values: ****p < 0.0001, compared with wild type by Mann-Whitney's *U* test. (B) $n = 5$ for control and $n = 6$ for *clc1Δ*. (D) $n = 9$ for control and *clc1Δ*.

Dnf1/Dnf2 and Cdc50-Drs2 complexes work in parallel during plasma membrane/cell wall repair.

Two of the plasma membrane repair factors, Pkc1 and Exo70, have negatively charged phospholipid binding domains required for plasma membrane binding [28,29]. Therefore, we hypothesized that phospholipid flippases remodel lipid composition at the damage site so that repair factors are recruited to the damage site. We first tested whether amount of Phosphatidyl Serine (PS) at the damage site is different in *lem3*Δ*cdc50*Δ cells compared to WT because PS is a substrate of Drs2-Cdc50 complex [30] and previous research implicated that Lem3-Dnf1/Dnf2 complex changes the amount of PS at plasma membrane by flipping phosphatidylethanolamine (PE) to the inner leaflet [31]. The signal of Lact-C2-mNeonGreen, a PS probe, accumulated at the damage site in both control and *lem3*Δ*cdc50*Δ cells after laser damage and that their accumulation kinetics were comparable (Fig. S3). We speculate that Lem3-Dnf1/Dnf2 and Cdc50-Drs2 complexes are involved in remodeling other substrates, such as PE, which we did not test because of cytotoxicity of the peptide, Ro09-0198, used as a PE probe [32]. We next evaluated the recruitment of Pkc1 and Exo70 to the damage site in flippase deleted mutants. We found that Pkc1-mNeonGreen was recruited to the damage site in both control and *lem3*Δ*cdc50*Δ cells, but it dissociated from the damage sites more promptly in *lem3*Δ*cdc50*Δ cells (Fig. S4). This result suggests that Lem3-Dnf1/Dnf2 and Cdc50-Drs2 complexes are required for retaining Pkc1 at the damage site. We also found that Exo70-mNeonGreen accumulation at the damage site was decreased in *cdc50*Δ and this phenotype was enhanced in *lem3*Δ*cdc50*Δ (Fig. 4B, C and Fig. S5). Therefore, consistent with SDS sensitivity (Fig. 4A), these results suggests that Exo70 accumulation at the damage site requires lipid remodeling by Lem3-Dnf1/Dnf2 and Cdc50-Drs2 complexes. Altogether, our findings propose a novel regulatory mechanism of local plasma membrane/cell wall repair, namely clathrin-flippases-exocyst.

4. Discussion

In this study, we aimed to reveal the protein trafficking mechanism and its cargos essential for plasma membrane repair in budding yeast. We propose that clathrin-mediated protein trafficking is involved in plasma membrane repair. We also identified phospholipid flippases as novel plasma membrane repair factors, whose recruitment to the damage site requires Sla1-dependent endocytosis. Deletion of flippases impaired the recruitment of the repair factor Exo70 to the damage site.

Clathrin-mediated protein trafficking pathways are evolutionarily conserved in eukaryotes [33]. Here, we show that clathrin-mediated trafficking is involved in plasma membrane repair in budding yeast. Further, we found that Dnf1 is recycled from bud tip to damage site via Sla1-dependent endocytosis upon laser damage (Fig. 3B). We speculate that this mechanism is conserved in higher eukaryotes. Indeed, consistent with our observation, clathrin-dependent endocytosis is activated in human cancer cells upon plasma membrane damage by cold atmospheric plasma [34].

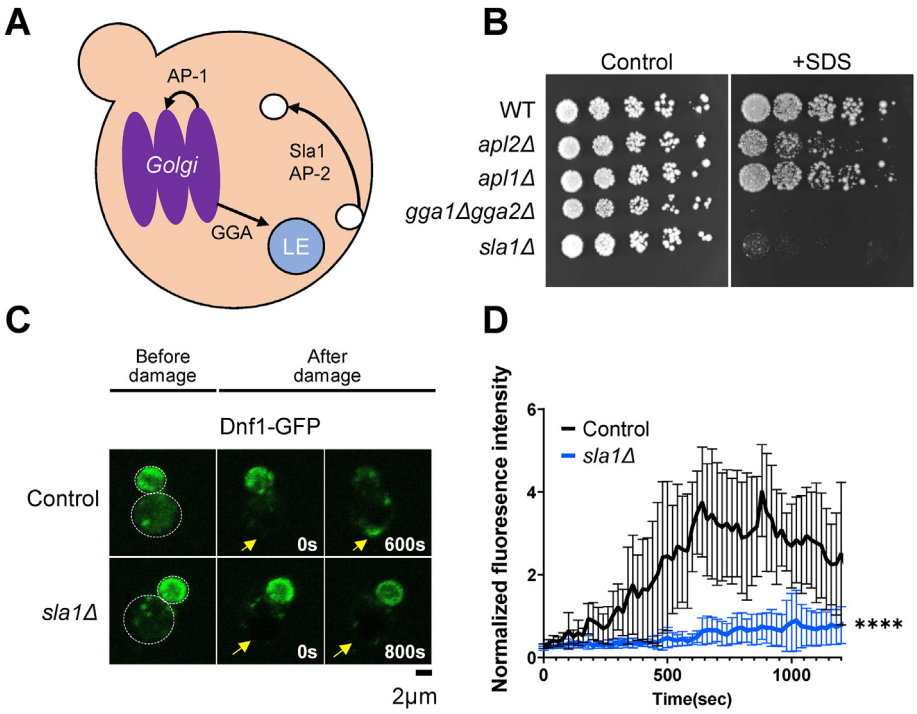

Fig. 3. Dnf1 is recycled from bud tip to the damage site via Sla1-dependent endocytosis. (A) Model of clathrin adaptor-dependent trafficking pathways. AP-1 mediates intra-Golgi recycling of TGN proteins. GGA mediates the trafficking of Golgi to late endosomes (LE). AP-2 and Sla1 mediate endocytosis. (B) Clathrin adaptor deleted mutants were grown in YPD or YPD + 0.02% SDS. 4-fold serial dilutions were spotted on each plate. (C) Dnf1-GFP in control and *sla1Δ* cells were monitored before and after 405 nm laser damage. (D) Fluorescence signal intensity at the damage site was normalized by whole cell fluorescence intensity. *P*-values: ****p < 0.0001 compared with control by Mann-Whitney's *U* test. $n = 6$ for control and *sla1Δ*.

However, further studies are needed to conclude that clathrin-mediated trafficking is a universal mechanism for plasma membrane repair in eukaryotes.

We found that two of the phospholipid flippase complexes, Lem3-Dnf1/Dnf2 and Cdc50-Drs2, accumulate at the damage site. Under unperturbed growth conditions, Lem3-Dnf1/Dnf2 localizes to the plasma membrane; Cdc50-Drs2 localizes to TGN [27]. Cdc50-Drs2 complex is required for TGN-associated protein trafficking pathways such as protein sorting into vesicles and exocytic vesicle formation [20,35,36]. These results suggest that these two complexes are involved in different cellular processes. However, our results suggest that Lem3-Dnf1/Dnf2 and Cdc50-Drs2 work redundantly during plasma membrane repair. Three lines of evidence support this conclusion: 1) both Dnf1 and Drs2 accumulate at the laser damage site (Fig. 2) and 2) *lem3*Δ*cdc50*Δ showed enhanced sensitivity to SDS-containing plates compared with single deletion mutants, *lem3*Δ and *cdc50*Δ (Fig. 4A), 3) accumulation of Exo70 at damage site was decreased in *lem3*Δ*cdc50*Δ compared with single gene deletion mutants, *lem3*Δ and *cdc50*Δ (Fig. 4B, C and Fig. S5). Thus, unlike in unperturbed growth conditions, Cdc50-Drs2 and Lem3-Dnf1/Dnf2 work in parallel during plasma membrane repair.

In this work, we showed that Lem3-Dnf1/Dnf2 and Cdc50-Drs2 is required for the recruitment of the repair factor, Exo70, to the damage site. We are considering two mechanisms for this result: 1) flippases activates Exo70 containing vesicle formation, 2) flippases promotes Exo70 binding to plasma membrane by remodeling the lipid composition at the damage site. The result that deletion of *CDC50* delays Exo70 accumulation (Fig. S5) supports the former possibility because Cdc50-Drs2 complex is involved in TGN-associated protein trafficking [20]. Further, deletion of *LEM3* and *CDC50* accelerates this phenotype while single deletion of *LEM3* does not impair the accumulation of Exo70 (Fig. 4B, C and Fig. S5) suggesting that the Lem3-Dnf1/Dnf2 complex at the damage site is also involved in Exo70 recruitment though its function could be complemented by the Cdc50-Drs2 complex at the damage site. Exo70 recruitment to the damage site is faster than that of Dnf1 and Drs2 (Fig. 2 and Fig. S5) suggesting that Cdc50-Drs2 and Lem3-Dnf1/Dnf2 at the damage site promote Exo70 retention at the damage site after its recruitment. Analogous to our finding, under unperturbed growth condition, Lem3-Dnf1/2 complex regulate the localization change of polarity factor, Cdc42, at the daughter cell by flipping neutral-charged phospholipid to inner leaflet [31]. Therefore, combining these observations, we propose following flippase-mediated Exo70 recruitment mechanisms. First, Golgi localized Cdc50-Drs2 complex facilitate Exo70 containing vesicle formation. After Exo70 recruitment to the damage site, Cdc50-Drs2 and Lem3-Dnf1/Dnf2 complexes at the damage site stabilize the association between Exo70 and plasma membrane by flipping phospholipid. Our study proposes a novel flippase-mediated plasma membrane repair mechanism involving evolutionally conserved protein complexes. This work serves as a basis for further studies aiming to reveal the conserved molecular mechanisms underlying the delivery and maintenance of plasma membrane repair factors.

Funding

This study was supported by MEXT/JSPS KAKENHI under Grant Number 20H03440 and JST-PRESTO JPMJPR1686 to K·K.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

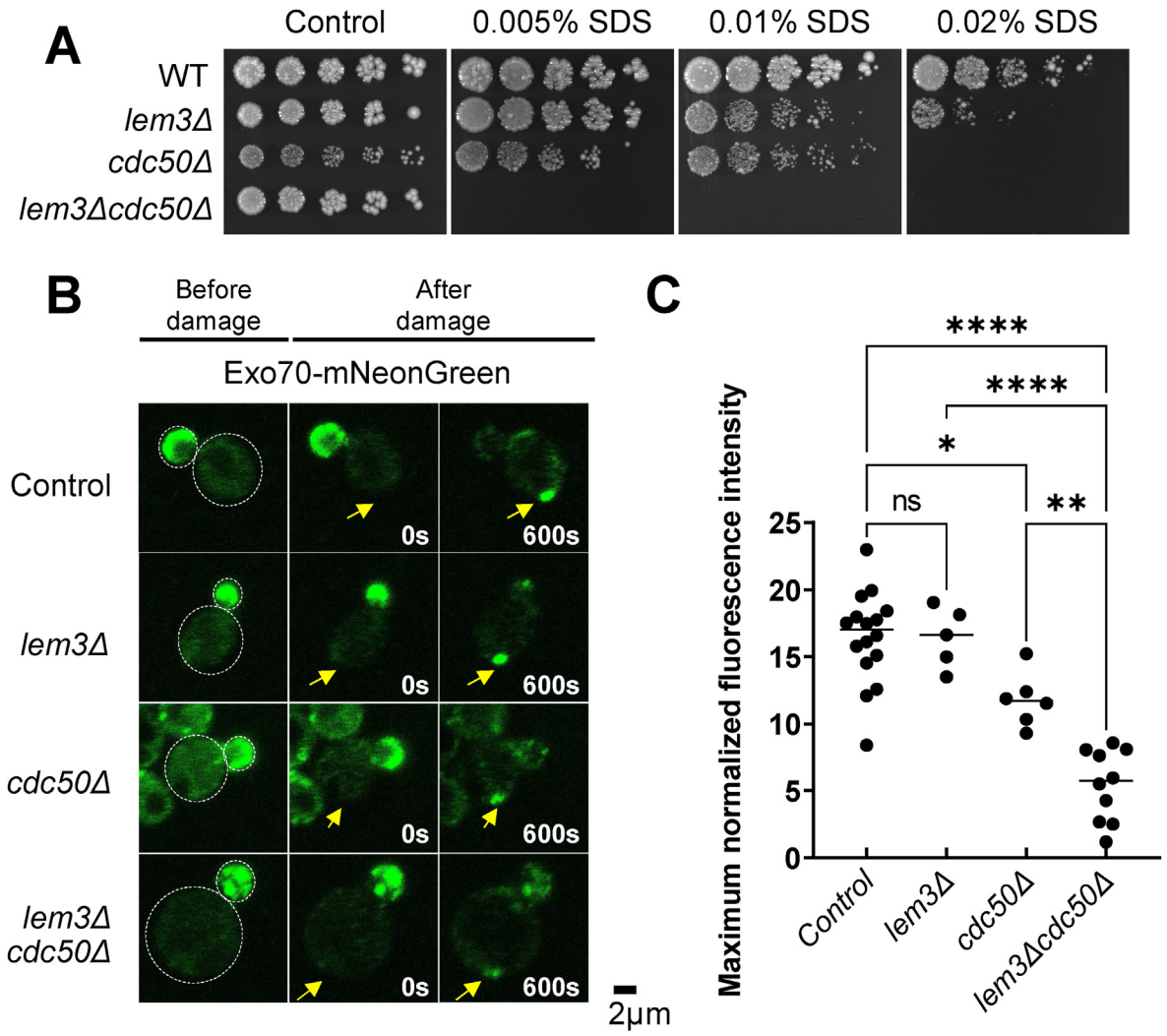

Fig. 4. Phospholipid flippases are required for Exo70 recruitment to the damage site. (A) Phospholipid flippase deleted mutants were grown in YPD or YPD + 0.02% SDS at 30 °C. 4-fold serial dilutions were spotted on each plate. (B) Exo70-mNeonGreen in control, *lem3*Δ, cdc*50*Δ, and *lem3*Δ*cdc50*Δ cells were monitored before and after 405 nm laser damage. Cell culture was performed at 30 °C. (C) Maximum fluorescence signal intensity at the damage site during time course was compared between samples. One-way analysis of variance (ANOVA) test and the post hoc Bonferroni test were performed. *P*-values: ****p < 0.0001 ***p < 0.001 **p < 0.01 *p < 0.05. $n = 16$ for control, $n = 5$ for *lem3Δ*, $n = 6$ for *cdc50Δ* and $n = 10$ for *lem3Δcdc50Δ*. Fluorescence signal intensity was normalized by whole cell fluorescence intensity.

Acknowledgement

We thank S. Sugiyama for providing yeast strains and technical advice. A portion of this work was performed with the help of OIST imaging section members. We thank K. Koizumi, T. Mochizuki, S. Komoto, and P. Barzaghi for technical assistance; K. Tanaka, Y. Moriyama, N.B. Razali, H. Barbee, S. Sugiyama and for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.03.129.

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