Title

Large-scale identification of plasma membrane repair proteins revealed spatiotemporal cellular responses to plasma membrane damage

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Abstract

Damage to the plasma membrane (PM) is common in all types of cells. PM repair processes, including exocytosis and endocytosis, are not mutually exclusive; rather, they collaborate to repair the wound. However, the temporal coordination between the repair processes remains poorly understood. Here, by large-scale identification and live-cell imaging of PM repair proteins, we analyzed the spatiotemporal PM damage responses in *Saccharomyces cerevisiae*. Of the 80 repair proteins identified, 72 proteins were previously unreported repair protein candidates. Among the observed repair processes, the polarized exocytosis and clathrin-mediated endocytosis (CME) are coupled at the damage site, with exocytosis predominating in the early stage of PM repair and CME predominating in the late stage of PM repair. Furthermore, we showed that CME at the growing bud site directs PM repair proteins with transmembrane domains to the damage site. We propose a model in which CME delivers repair proteins with transmembrane domains between the growing bud site and the damage site. This study provides a functional catalog of PM repair proteins and insights into spatiotemporal cellular responses to PM damage.

Introduction

The plasma membrane (PM) functions as a defensive barrier in all types of cells to protect cellular components from extracellular stimuli. PM frequently experiences physiological and pathological damages, such as eccentric contraction-induced injuries, migration-induced injuries, pore formation by bacterial toxins, or invasion by cancer cells (1, 2). Cells undergo cell lysis if the damaged PM is not repaired. Deficits in PM repair are linked to multiple diseases, including limb-girdle muscular dystrophy (3) and Scott syndrome (4, 5). Therefore, virtually all cells have PM repair machinery.

In eukaryotes, the influx of Ca^{2+} triggers PM repair (1). Upon Ca^{2+} influx, fundamental cellular mechanisms, including exocytosis (6-10), endocytosis (11, 12), membrane shedding by ESCRT complex (13, 14), and the constriction forces by actin cytoskeleton (15-18) are directed to the damage site to reseal the wound. These initial repair processes usually occur within 1 min after PM damage (19). After the resealing of the damaged PM, cells restructure the damaged PM (19-21). Restructuring is defined as the process by which cells modify the damaged PM to restore PM homeostasis and normal cell function (19-21). These repair processes are not mutually exclusive, and they collaborate to repair the damaged PM (22). However, the temporal coordination between the PM repair processes remains poorly understood.

Both resealing and restructuring of the damaged PM are mediated by the proteins, which accumulate at the damage site. Here, we defined them as PM repair proteins. PM repair proteins include ESCRT, annexins, and dysferlin in mammalian cells (13, 23-25). Using budding yeast *Saccharomyces cerevisiae* as a model, we previously identified evolutionarily conserved repair proteins, including protein kinase C, exocyst, and phospholipid flippases (26, 27). These repair proteins accumulate at the damage site at the appropriate time and proceed with the repair processes. Therefore, identifying repair proteins and observing their movements after PM damage is an important step toward understanding the temporal PM repair processes.

Here, we performed proteome-scale screening of PM repair proteins using yeast GFP-tagged libraries and laser-induced injury assay in budding yeast. We identified 80 repair protein candidates, including 72 previously unreported candidates. Among the observed repair processes, we showed that polarized exocytosis and clathrin-mediated endocytosis (CME) are coupled at the damage site, with exocytosis predominating in the early stage of PM repair and CME predominating in the late stage of PM repair. We also showed that CME delivers repair proteins with transmembrane domains (TMDs) from the growing bud site (bud tip) to the damage site, presumably contributing to the restructuring of the damaged PM. These results provide insights into the temporal dynamics of coordinated cellular responses to PM damage.

Results

Proteome-scale identification of SDS-responsive proteins

To identify the PM repair proteins, we performed a two-step screening. First, we aimed to identify the proteins that change localization in response to SDS treatment, which induces local PM/cell wall damage to budding yeast (28). We used a yeast C-terminally GFP-tagged library (4159 ORFs) (29) and an N-terminally sfGFP-tagged library (N' Swat library) (5569 ORFs) (30, 31) comprising 86% of the yeast proteome (5718 ORFs in total) (Fig. 1A). We fixed the cells with paraformaldehyde after 1 hour of SDS treatment, in which condition the one of the known repair proteins, Pkc1-GFP, changes its localization (Fig. 1A). We successfully imaged the signal of 9181 proteins fused with GFP or sfGFP (5609 ORFs in total). We assessed the fluorescence signals of 5609 proteins in normal and SDS-treated conditions by visual inspection of the images. We identified 562 proteins whose fluorescence signal pattern changed after SDS treatment (Fig. 1B and Data S1). The hits included the localization changes of proteins, structural changes of organelles such as mitochondrial fragmentation, and foci/puncta formation in response to SDS treatment. In addition to Pkc1, we identified previously reported repair proteins, such as Rom2

98 and Dnf1, as screening hits (26, 27) (Data S1). Gene Ontology (GO) analysis of the screening hits
99 revealed enrichment for proteins involved in the actin filament organization (Fig. 1C). This is
100 consistent with the previous study that SDS treatment remodels the cell polarity and actin
101 cytoskeleton (26). Based on the reported subcellular localization of proteins in normal growth
102 conditions (29), we categorized the screening hits into six major classes and several minor ones
103 representing less than ten proteins (Fig. 1D and fig. S1, see Materials and Methods). We found
104 that proteins that form puncta/foci after the SDS treatment include translation factor Gcd7-GFP
105 (Fig. 1D), which forms foci in response to glucose deprivation (32). These observations suggest
106 that SDS treatment induces stress responses, including those associated with PM/cell wall damage
107 responses in budding yeast.
108
109 **Laser-induced PM/cell wall damage assay identified 80 repair protein candidates**
110 Our group previously developed a laser-induced PM/cell wall injury assay under live single-cell
111 conditions (laser damage assay) (26, 33). We performed the laser damage assay to identify the
112 PM repair proteins and observe their movements after PM/cell wall damage (Fig. 2A). The N-
113 terminally sfGFP-tagged library is expressed under exogenous *NOP1* promoters, while the C-
114 terminally GFP-tagged library is expressed under endogenous promoters (29, 30). To assess the
115 proteins with endogenous expression levels, we selected the 234 screening hits from the C-
116 terminal library as targets for the laser damage assay. During 25 min of observation at 30-second
117 intervals, we identified 90 proteins whose localization changed in response to laser damage (Fig.
118 2B). We categorized the proteins into four classes based on their localization changes (Fig. 2C).
119 Three of the puncta-forming proteins, Dna2, Dot6, and Gcd7, form puncta in response to cellular
120 stresses (32, 34). The internalization of PM proteins (Dip5, Ina1, Ftr1, and Tpo1) is consistent
121 with the fact that endocytosis of PM proteins contributes to cell adaptation to environmental stress
122 (35). In addition, the transcription factors, Msn2 and Crz1, translocate from the cytoplasm to the
123 nucleus after laser damage (Fig. 2B). Msn2 and Crz1 are activated under various stress conditions
124 (36). Thus, the localization changes of puncta-forming proteins and transcriptional factors suggest
125 common stress responses to laser damage and other cellular stresses. The most frequently
126 observed localization changes were the accumulation at the damage site (Fig. 2B). We defined
127 them as repair protein candidates, given their presumed involvement in the PM/cell wall repair
128 process.
129 To gain further insight into the repair protein candidates, we classified them into four categories
130 based on their subcellular localization under normal growth conditions (Fig. 2C). The largest
131 category is bud-localized proteins (47 proteins) (Fig. 2C), which is consistent with a previous
132 study showing that bud-localized proteins accumulate at the damage site in budding yeast (26).
133 The repair protein candidates in this category include ten proteins that have transmembrane
134 domains (TMDs), including phospholipid flippases (Dnf1/Dnf2) and osmosensor proteins (Slg1,
135 Sho1) (Fig. 2C). Proteins with TMDs are transported to the PM as cargoes of secretory vesicles
136 (37). Thus, this result suggests that secretory vesicles targeted to the damage site provide repair
137 proteins with TMDs to the damage site. The second largest category is actin-localized proteins
138 (25 proteins). This is consistent with the GO analysis that actin-binding proteins and proteins
139 involved in actin-related biological processes are enriched in the repair protein candidates (Fig. 2,
140 D and E). The proteins in this category include endocytic proteins, suggesting that endocytosis
141 occurs at the damage site. Proteins that bind actin cables, such as Abp140, are included in this
142 category, suggesting that actin cables are formed at the damage site. In addition, proteins involved
143 in cell wall synthesis/maintenance (Flc1, Dfg5, Smi1, Skg1, Tos7, and Chs3) are identified as
144 repair protein candidates (Data S2). These results are consistent with previous studies of PM/cell
145 wall damage repair in budding yeast and human cells (11, 26, 38, 39).
146

147 **The temporal order of the Pkc1 accumulation, polarized exocytosis, and CME at the**
148 **damage site**
149 To understand when repair proteins accumulate at the damage site, we defined their accumulation
150 times as the time when the fluorescence intensity at the damage site exceeded the threefold
151 standard deviation (3xSD) above the non-damaged site for at least two consecutive time frames (1
152 min). We also defined dispersion time when the fluorescence intensity at the damage site becomes
153 less than that of the non-damaged site plus 3xSD for at least 1 min (Data S2).
154 The defined accumulation times raised the possibility that the Pkc1 pathway proteins accumulated
155 at the damage site earlier than exocytosis regulators and exocytic cargoes with transmembrane
156 domains (Dnf1, Dnf2, and Slg1) (Fig. S2). To verify this observation, we performed the laser
157 damage assay in the cells expressing Exo70-mNeonGreen (mNG) and Pkc1-mScarlet-I (mSc-I),
158 and the cells expressing Pkc1-sfGFP and Dnf1-mSc-I (Fig. 3A and B). Exo70 is one of the
159 subunits of the exocyst complex, serving as a polarized exocytosis marker. The fluorescence-
160 tagged Pkc1 accumulated at the damage site earlier than Exo70-mNG and Dnf1-mSc-I (Fig. 3, A
161 and B). These results suggest that the Pkc1 accumulation at the damage site occurs earlier than the
162 polarized exocytosis of transmembrane proteins.
163 Although not all exocyst subunits (eight subunits) were identified in this work, all the mSc-I-
164 tagged exocyst subunits showed comparable fluorescence intensity changes with Exo70-mNG at
165 the damage site and at the bud tip (Fig. S3, A to G). These results suggest that all subunits of the
166 exocyst complex accumulate at the damage site simultaneously.
167 The CME markers, Sla1-GFP and Abp1-GFP (40), exhibited repeated short stays and
168 reaccumulation at the damage site within 5 min to 25 min after laser damage (Fig. S4, A to C).
169 These fluctuating movements of Sla1 and Abp1 are consistent with the previous studies (41, 42).
170 Furthermore, Sla1-mNG and Abp1-mSc-I showed comparable fluctuating accumulation patterns
171 in the same cell (Fig. S4D). These results suggest that CME repeatedly occurs at the damage site
172 from around 5 min to 25 min after laser damage.
173 To understand the temporal order of polarized exocytosis and CME at the damage site, we
174 performed the laser damage assay in the cells expressing Exo70-mNG/Dnf1-mNG and Ede1-
175 mSc-I (Fig. 3, C and D). Ede1 is one of the earliest proteins recruited to endocytic sites, serving
176 as a CME marker (43, 44). Exo70-mNG/Dnf1-mNG and Ede1-mSc-I accumulated at the damage
177 site from 5 to 35 min (Fig. 3, C and D). The signals of Exo70-mNG/Dnf1-mNG peak within 20
178 min after the damage, while Ede1-mSc-I peaks 20 min after the damage (Fig. 3, C and D). These
179 results suggest that CME and polarized exocytosis occur simultaneously at the damage site, but
180 that polarized exocytosis predominates in the early stage of the PM/cell wall damage response,
181 while CME predominates in the late stage of the PM/cell wall damage response.
182
183 **Knockout mutants of CME are sensitive to PM/cell wall stresses**
184 To identify the biological processes required for cell survival after PM/cell wall stress, we
185 performed growth screening of repair protein knockout mutants in PM/cell wall stress conditions
186 (Fig. S5A). We spotted the same number of yeast cells in YPD media, YPD+0.01% SDS media,
187 YPD+25 μg/ml calcofluor white (CFW), and YPD media in 37 ℃ (Heat stress) and incubated for
188 3 days (Fig. S5A). CFW binds to chitin in the cell wall, inducing cell wall damage (45). Heat
189 stress modifies PM structures (46). The growth assay showed that six knockout mutants of CME
190 proteins, *rvs167*Δ, *sla1*Δ, *sla2*Δ, *end3*Δ, *las17*Δ, and *vrp1*Δ, are sensitive to all the stress
191 conditions (Fig. S5B). We knocked out these genes using the WT strain in our laboratory and
192 confirmed that these mutants are sensitive to SDS (Fig. S5C). These results led us to focus further
193 on the CME functions associated with PM/cell wall damage repair.
194
195 **CME proteins are required for polarized exocytosis at the damage site**

196 Previous studies showed that endocytosis is involved in PM damage repair by directly resealing
197 the PM by removing the damaged pores (47) or by restructuring the PM after resealing in
198 mammalian cells (20, 21, 48). We showed that CME occurs at the damage site from 5 to 45 min
199 after laser damage in budding yeast (Fig. 3, B and C). This supports the possibility that CME
200 restructures the PM after resealing, which usually occurs within 1 min after the damage (20).
201 Another potential mechanism for restructuring the damaged PM is exocytosis, which delivers PM
202 proteins and lipids to the damage site. Our previous work showed that the polarized exocytosis
203 machinery is directed from the bud tip to the damage site in response to laser damage in budding
204 yeast (26). Given that CME and polarized exocytosis constitute a coupled transport cycle in
205 budding yeast (49), we reasoned that CME positively regulates polarized exocytosis at the
206 damage site, thereby restructuring the damaged PM.
207 To test this idea, we performed the laser damage assay in SDS-sensitive endocytic mutants
208 (*sla1*Δ, *end3*Δ, *vrp1*Δ, and *rvs167*Δ). We used two exocytosis markers, type V myosin (Myo2)
209 and the exocyst subunit (Exo70), fused to sfGFP and mNG, to assess the exocytosis activity at the
210 damage site. The effect of deleting the CME genes on the expression levels of Myo2-sfGFP and
211 Exo70-mNG before laser damage was minimal (Fig. S6, A and B). The accumulation of Myo2-
212 sfGFP and Exo70-mNG at the damage site was impaired in *sla1*Δ, *end3*Δ, and *vrp1*Δ (Fig. 4, A
213 and B, fig. S6, A and B). Moreover, we also found that the dispersion of Myo2-sfGFP and Exo70-
214 mNG from the bud tip was partially inhibited in CME mutants (Fig. 4, A and B, fig. S6, A and B).
215 The observed weak phenotype of *rvs167*Δ for the accumulation and dispersion of Myo2-sfGFP
216 and Exo70-mNG is consistent with the previous study that exocytosis is not impaired in *rvs167*Δ
217 (49).
218 Our previous work also showed that the actin nucleator formin Bni1 and the exocyst subunit Sec3
219 are degraded in response to SDS treatment (26). To test the possibility that CME is required for
220 the decrease of Bni1 and Sec3 after SDS treatment, we performed immunoblotting of Bni1-
221 13xMyc and Sec3-GFP before and after SDS treatment (Fig. S7, A and B). Protein levels of Bni1-
222 13xMyc and Sec3-GFP were decreased in WT, *rvs167*Δ, and *end3*Δ after SDS treatment (Fig. S7,
223 A and B). Given that *end3*Δ impaired the accumulation of Myo2-sfGFP and Exo70-mNG at the
224 damage site (Fig. 4, A and B), these results suggest that CME is dispensable for the decrease of
225 Bni1 and Sec3 after SDS treatment. Altogether, these results suggest that CME is involved in the
226 direction of the Exo70-mNG and Myo2-sfGFP to the damage site in a Bni1- and Sec3-
227 degradation-independent manner.
228
229 **CME at the bud tip directs repair proteins with TMDs to the damage site**
230 Although the requirement of Rvs167 for the Pkc1 accumulation and polarized exocytosis at the
231 damage site is minimal (Fig. 4, A to C), *rvs167*Δ is sensitive to SDS (Fig. S5C). This raised the
232 possibility that CME plays additional roles, such as membrane protein trafficking, in PM repair.
233 We found that CME proteins, including Apl1, Ede1, and Ent1, changed their localization from the
234 bud neck to the bud tip within 10 min after laser damage (Fig. S8A). The fluorescence intensity
235 changes of CME proteins at the bud tip at 10 min after laser damage were higher than those of
236 other repair protein candidates (Fig. S8B). Consistent with these results, the Ede1-mSc-I signal at
237 the bud tip increased within 10 min after laser damage, and then the Ede1-mSc-I signal at the
238 damage site increased (Fig. 5A). These results suggest that CME occurs at the bud tip within 10
239 min after laser damage. We also found that Dnf1-mNG disappeared from the bud tip following
240 the recruitment of Ede1-mSc-I to the bud tip (Fig. 5, A and B). The disappearance from the bud
241 tip and the accumulation at the damage site of Dnf1-mNG were impaired in *rvs167*Δ (Fig. 5, B
242 and C, and fig. S9). Furthermore, the disappearance from the bud tip and the accumulation at the
243 damage site of repair proteins with TMDs (Slg1-sfGFP and Sho1-GFP) and representative
244 endocytic recycling cargo, mNG-Snc1, were impaired in *rvs167*Δ (Fig. 5, D to F and fig. S10, A

to C). These results support our idea that CME directs repair proteins with TMDs from the bud tip to the damage site.

mNG-Snc1 is retargeted from the damage site to the bud tip

The fluorescence intensity of accumulated repair proteins with TMDs at the damage site gradually decreases approximately 15 min after laser damage (Fig. 3D and fig. S10, A and C). Using mNG-Snc1 as a model, we investigated the destination of repair proteins after PM repair. To avoid observing the mNG-Snc1 that is newly synthesized after laser damage, we transiently expressed mNG-Snc1 under the control of the galactose-inducible promoter (Gal1pr) before laser damage (Fig. 6A). After 1 hour of mNG-Snc1 expression in galactose media, we transferred the cells to glucose media to stop the expression (Fig. 6A). To minimize the effect of changing the carbon source, we further incubated the cells in glucose media for at least 3 hours prior to laser damage (Fig. 6A). The mNG-Snc1 accumulated at the damage site approximately ~10 min after laser damage, gradually disappearing after the colocalization with Ede1-mSc-I (Fig. 6A and B). The mNG-Snc1 signal at the bud tip decreased to 10-53% 22 min after laser damage (Fig. 6C and D). The normalized mNG-Snc1 signal at the bud tip recovered to 54-88% 50 min after laser damage. (Fig. 6, C and D). These results suggest that mNG-Snc1 is redirected to the bud tip after PM repair.

Discussion

There has been a growing interest in the mechanisms underlying PM repair, partly due to their association with human diseases and cellular aging (3-5, 28). PM repair proteins, which accumulate at the damage site, play critical roles in PM repair. In this study, by large-scale identification of PM repair proteins and single- and dual-color live-cell imaging of repair proteins, we analyzed spatiotemporal PM repair processes in budding yeast. We propose a model in which CME at the bud tip and at the damage site delivers repair proteins with TMDs between the bud tip and the damage site, allowing the cell to restructure the damaged PM and to resume growth after PM repair (Fig. 7).

Two-step visual screening for PM repair protein identification

By combining proteome-scale visual screening using yeast GFP libraries and the laser damage assay, we identified 80 repair protein candidates (Fig. 2B and Data S2). Strikingly, 72 out of 80 repair protein candidates were not previously reported to accumulate at the damage site in budding yeast (26, 27). The unreported repair protein candidates include uncharacterized proteins, such as Sap1 and Ypr089w (Data S2). Characterizing these proteins may expand our understanding of PM repair mechanisms.

We selected the screening hits from the C-terminally GFP-tagged library as targets for the laser damage assay. Although this is a reasonable approach to evaluating the proteins from the endogenous expression level, it overlooks the potential repair proteins in the N-terminally sfGFP-tagged library. Around 23% of ORFs only exist in the N-terminal library, and 11% of yeast ORFs show different localization from that of the C-terminal library (31). For example, sfGFP-Snc2, but not Snc2-GFP, changes localization in response to SDS treatment because of the mislocalization of Snc2-GFP in the vacuole. Because Snc2 is a homolog of Snc1, Snc2 is a potential repair protein. In addition, 48 out of 80 repair proteins identified in this work were hits in the N-terminally sfGFP-tagged library screening (Table S1 and S2). Screening hits from the N-terminally sfGFP-tagged library can also be a useful resource for further identification of repair proteins.

The screening is unable to identify proteins whose localization remains unaltered in response to SDS treatment. For example, we could not identify Cdc50-Drs2, which accumulates at the damage site induced by a laser (27). A recent study demonstrated the different cellular responses

295 between focal (laser damage) and diffuse (streptolysin-O (SLO) treatment) PM damage (6). Some
296 proteins may be overlooked in the screening due to the intrinsic differences between SDS
297 treatment and the laser damage assay. Using different stresses to induce PM damage may identify
298 additional PM repair proteins. In addition, the screening did not identify ESCRT proteins as hits.
299 The tagging of fluorescent proteins occasionally interferes with the functions of the tagged
300 proteins (50). Specifically, the exogenous expression of fluorescent-tagged ESCRT subunits can
301 impair their functions (51, 52). The screening may have overlooked some of these proteins,
302 possibly including ESCRT proteins.
303 We performed live-cell imaging of repair proteins and defined accumulation times of repair
304 proteins (Fig. S2 and Data S2). This dataset provides an overview of repair protein accumulation.
305 However, it should be noted that the accurate relative accumulation timing of repair proteins
306 should be determined by multi-color imaging of repair proteins in the same cells, because of cell-
307 cell variabilities, the GFP-tagging effects on the cells, and the small sample size of the screening.
308 These datasets will form the basis for future hypothesis-driven studies.
309
310 **Coordination of polarized exocytosis and CME**
311 We previously showed that formin Bnr1 forms the actin cable at the damage site (26). Consistent
312 with this, we identified Bnr1-interacting proteins, including Bud6 and Smy1, as repair proteins
313 (Data S2). Because actin cables mediate polarized exocytosis and secretory vesicle trafficking
314 (53), these results further support our model that exocytosis delivers repair proteins with TMD at
315 the damage site (Fig. 7).
316 We showed that polarized exocytosis and CME occur simultaneously at the damage site between
317 approximately 5 and 35 min after laser damage (Fig. 3, C and D). Given that the resealing of the
318 damaged PM generally occurs within 1 min (20), this result implies that polarized exocytosis and
319 CME are involved in the restructuring of the damaged PM rather than the resealing of it. The
320 coupling of endocytosis with polarized exocytosis is observed in multiple cell types, including at
321 the growing tip of budding yeast, the growing tip of pollen tubes, and synapses in neurons (54).
322 Polarized exocytosis and CME at the damage site may regulate the PM tensions and amount of
323 lipids and PM proteins in a manner analogous to these cells.
324 Our results suggest that the activity of CME and polarized exocytosis at the damage site changes
325 over time, with exocytosis predominating within 20 min after laser damage and CME
326 predominating 20 min after laser damage (Fig. 3, C and D). This is consistent with the recent
327 study in human cells (21). A previous study showed that polarized exocytosis activates CME in
328 budding yeast (49). Rab GTPase Sec4, which regulates polarized exocytosis, also activates
329 endocytosis by overriding Sla1 inhibition of Las17 (49). We could not identify Sec4 as a repair
330 protein candidate because C-terminally GFP-tagged Sec4 is not in the library, probably due to the
331 loss of function of C-terminally tagged Sec4. However, we found that Sec2-GFP, the guanine
332 nucleotide exchange factor (GEF) of Sec4, accumulates at the damage site (Data S2). Sec4 and
333 Sec2 are localized to the secretory vesicles (55, 56). Therefore, secretory vesicles that are targeted
334 to the damage site may accumulate Sec2 and Sec4, leading to the activation of CME. The
335 switching of activities between polarized exocytosis and CME may contribute to restoring PM
336 homeostasis after the damage.
337 Polarized exocytosis at the damage site is inhibited in CME mutants (Fig. 4, A and B, and fig. S6,
338 A and B). Given that CME and polarized exocytosis occur simultaneously (Fig. 3, C and D),
339 CME may activate polarized exocytosis at the damage site, such as by regulating the PM tension
340 (57) around the damage site. Moreover, Myo2-sfGFP and Exo70-mNG were partially retained at
341 the bud tip after laser damage in CME mutants (Fig. 4, A and B, and fig. S6, A and B). These
342 results raise the possibility that CME is involved in targeting Exo70 and Myo2 from the bud tip.
343 At the bud tip, CME may be involved in the dispersion of Exo70 and Myo2 via upstream
344 regulators, such as Rho3.

345
346 **CME functions for PM repair in budding yeast**
347 Previous studies showed that endocytosis actively occurs at the damage site to repair the damaged
348 PM (11, 12, 20). Surprisingly, our results suggest that CME occurs not only at the damage site but
349 at the bud tip within 10 min after laser damage. In *rvs167*Δ, in which exocytosis inhibition at the
350 damage site is minimal (Fig. 4, B and C), repair proteins with TMDs at the bud tip fail to
351 accumulate at the damage site (Fig. 5, B to F). These results are consistent with our idea that
352 CME at the bud tip directs repair proteins with TMDs to the damage site (Fig. 7). Consistent with
353 our results, dysferlin-containing vesicles increase in response to PM damage in muscle cells (25,
354 58). Dysferlin has a TMD and functions as a PM repair protein (25, 58, 59). Delivery of repair
355 proteins with TMDs from non-damaged sites to the damage site by endocytosis may occur in a
356 wide range of eukaryotic species.
357 We showed that 54-88% of the normalized mNG-Snc1 signal at the bud tip is recovered 50 min
358 after the damage (Fig. 6, C and D). Because Snc1 is a CME cargo and it colocalizes with Ede1-
359 mSc-I at the damage site, it is presumably recovered from the damage site via CME. Previous
360 studies showed that macropinocytosis and CME restructure the damaged PM in human cells (20,
361 21). However, it is not clear whether the cargo proteins are recycled or degraded. We propose that
362 CME terminates the PM damage responses by removing the repair proteins with TMDs from the
363 damage site and resuming the growth by retargeting them to the bud tip (Fig. 7). Further
364 confirmation of the proposed model requires other experimental strategies, such as the photo-
365 bleaching and photo-conversion of repair proteins, and the use of other repair proteins with
366 TMDs.
367
368 Here, we showed spatiotemporal cellular responses after PM damage in budding yeast by large-
369 scale identification of repair proteins and their live-cell imaging. Despite the limitations
370 mentioned above, our datasets provide the first functional catalog for PM repair proteins. Because
371 some of the PM repair proteins identified in this study and CME mechanisms are evolutionarily
372 conserved, this work may serve as a basis for future studies, including those to be conducted in
373 mammalian cells.
374
375 **Materials and Methods**
376 **Media and strains**
377 Standard procedures were used for DNA, *E. coli*, and yeast genetic manipulation. Yeast
378 transformations were performed using the lithium acetate method. A PCR-based procedure was
379 used for gene deletion. The deletion of the expected locus was confirmed by colony PCR (60).
380 Yeast cells were cultured in YPD media (1% yeast extract, 2% bacto peptone, and 2% glucose)
381 unless otherwise indicated. SD media (yeast nitrogen base (6.7 g/liter) without amino acids, L-
382 adenine (550 mg/liter), L-arginine (280 mg/liter), L-alanine (280 mg/liter), L-asparagine (280
383 mg/liter), L-aspartic acids (280 mg/liter), L-cysteine (280 mg/liter), glycine (280 mg/liter), L–
384 glutamic acids (280 mg/liter), L-glutamine (280 mg/liter), L-isoleucine (280 mg/liter), L-lysine
385 (280 mg/liter), L-phenylalanine (280 mg/liter), L-proline (280 mg/liter), L-serine (280 mg/liter), L-
386 threonine (280 mg/liter), L-tyrosine (280 mg/liter), L-valine (280 mg/liter), leucine (530 mg/liter),
387 methionine (86 mg/liter), histidine (86 mg/liter), uracil (22 mg/liter), myo-inositol (100 mg/liter),
388 and *p*-aminobenzoic acid (3 mg/liter) (pH 5.5)) were used for the laser damage assay. Yeast
389 culture was performed at 25°C unless otherwise indicated. The yeast strains, yeast libraries, and
390 plasmids used in this study are listed in Tables S1 to S3.
391 For the mNG-Snc1 expression experiments in Fig. 6, we grow cells in SD media containing 2%
392 raffinose instead of glucose overnight. Then, we induced the expression of mNG-Snc1 by adding
393 3% galactose. After one hour of growth in galactose media, the cells were centrifuged at 5000xg
394 for 5 min, and the spun-down cells were washed twice with SD media containing 2% glucose. We

395 transferred the spun-down cells to SD media containing 2% glucose. The cells were incubated for
396 at least 3 hours before the laser damage assay.
397
398 **GFP screen for SDS damage response**
399 GFP strains were spotted onto the YPD plates from 96-well plates using a pin replicator and
400 incubated at 25℃. After 3 days, the colonies were inoculated into 200 µl of YPD media and
401 incubated overnight to saturation. After mixing, 3 µl of the saturated culture was transferred to 1
402 ml of YPD media in deep well plates and incubated for 4-5 hours. 500 µl of the cultures were
403 transferred to the new deep well plates. 10 µl of 1% SDS was added to 500 µl of the cultures and
404 incubated at 25℃ for 1 hour. The cells were centrifuged at 2000xg for 2 min. The cells were fixed
405 by 300 µl of 4% PFA in YPD for 30 min at room temperature. The fixed cells were centrifuged at
406 2000xg for 2 min. Cells were washed twice with PBS and centrifuged at 2000xg for 2 min. The
407 supernatant was removed between each wash. Cells were kept at 4℃ until imaging. Cells were
408 imaged with an LSM880 confocal microscope using a 20x air objective lens with an airyscan
409 detector for GFP fluorescence (Ex 488/Em522). Maximum intensity projections of z-stack images
410 are shown.
411
412 **Categorization of localization changes in response to SDS treatment**
413 We manually reviewed the acquired images using Zen Blue edition (Zeiss) or Fiji software (61)
414 and compared the fluorescence signals of GFP-tagged proteins in normal and SDS treatment
415 conditions. We identified the proteins whose fluorescence signal pattern changes between the two
416 conditions. We categorized the screening hits based on reported protein localization (29). We first
417 categorized the bud tip- or the bud neck-localizing proteins as "From bud tip/neck". Among the
418 uncategorized remaining screening hits, the actin-localizing proteins were categorized as "Actin".
419 Among the remaining uncategorized screening hits, cell periphery-localizing proteins were
420 categorized as "From cell periphery." Among the remaining uncategorized screening hits,
421 nucleus-localizing proteins were categorized as "Nucleus" or "Nucleus to the cytoplasm". We
422 categorized Urc1 as "Nucleus to cytoplasm" because its signal dispersed to the cytoplasm in an
423 SDS treatment condition. Other proteins in the "Nucleus" showed stronger nucleus localization in
424 the SDS treatment conditions. Among the remaining uncategorized screening hits, we categorized
425 mitochondria-localizing proteins as "Mitochondria". Among the remaining uncategorized
426 screening hits, we categorized spindle pole-localizing proteins as "Spindle pole." All proteins in
427 the "Spindle pole" showed stronger dot-like structures in the SDS treatment condition. Among the
428 remaining uncategorized screening hits, we categorized proteins that show puncta or foci in SDS
429 treatment conditions as "Puncta/foci." We categorized Gcn2, Ato3, Sun4, Csi2, Sps4, and
430 Ypr089w as "Puncta/foci dispersed" because they showed weaker foci or puncta signals in SDS
431 treatment conditions. We categorized Tul1, Emp24, Hor7, Scw10, and Ynl019c as "From
432 vacuole" because they showed decreased vacuole signal in the SDS treatment conditions. We
433 categorized Prm5 as "to vacuole" because its vacuole signal increases. We categorized Yps1 and
434 Msc1 as "cytoplasm to ER" because they localize to the ER, and their ER signal increased in SDS
435 treatment conditions. In this study, we make use of the protein localization data from the
436 Saccharomyces Genome Database (SGD) and (29). All results of this screening are shown in Data
437 S1.
438
439 **Laser damage assay**
440 Yeast cells were grown in SD medium at 25°C until the culture reached an OD600 of 0.1-0.4. We
441 diluted the yeast culture and further incubated the yeast cells in SD medium for 3-8 hours at 25˚C.
442 We took 1 mL of the culture and centrifuged it at 500xg for 5 min to spin the cells down. We took
443 5-10 µL of the cell suspension and placed it onto SD medium + 2.2% agarose bed. A

444 Concanavalin A (ConA) (Nacalai Tesque) coated glass slip was placed on the cells prior to
445 imaging.
446 Cells were observed with A1R (Nikon). A1R was equipped with a CFI Apochromat TIRF
447 60×/1.49 oil objective lens (Nikon). GFP, mNG, and sfGFP were excited by a 488 nm laser, and
448 the fluorescence that passed a 525/50 nm band-pass filter was detected with a GaAsP detector.
449 mSc-I was excited by a 561 nm laser, and the fluorescence that passed a 595/50 nm band-pass
450 filter was detected with a GaAsP detector. For the laser damage assay, the 405 nm laser was
451 irradiated to a circle of 0.5 μm diameter in a cell periphery. The laser power was set between 30%
452 and 70%, depending on the fluorescence-tagged proteins. We determined the laser power
453 sufficient for repair protein recruitment at the damage site in control cells without resulting in cell
454 lysis during the experiments. At least ~80% of cells are viable during the experiments, otherwise
455 indicated. Only the cells that survive after laser damage are quantified. To compare the
456 accumulation of repair proteins between different strains, we set the exact same laser power and
457 microscopy.
458
459 **Categorization of localization changes in response to laser damage**
460 We categorized proteins that accumulate at the damage site as “Damage site”. These proteins are
461 defined as repair protein candidates. Among the remaining uncategorized proteins, we categorized
462 cell periphery-localizing proteins as “PM to cytoplasm”. Among the remaining uncategorized
463 proteins, we categorized Msn2 and Crz1 as "Nucleus" because they changed localization from the
464 cytoplasm to the nucleus. Among the remaining uncategorized proteins, we categorized Dot6,
465 Dna2, Msc1, and Gcd7 as "Puncta" because they formed punctate structures in response to laser
466 damage. The repair protein candidates identified in this study are listed in Data S2.
467
468 **Quantification of fluorescence signal for laser damage assay**
469 The quantification of the fluorescence signal from the laser damage assay was performed as
470 described previously (27). For the large-scale identification of repair protein candidates, we
471 selected the region of interest (ROI) around the whole cell, the damage site, and the bud tip of the
472 cells. We manually move the ROIs as the cell moves so that the ROIs remain at the same position
473 in the cell. We set the ROIs and manually selected them for quantification. The fluorescence
474 intensity at the damage site and the bud tip were normalized by the fluorescence intensity of a
475 whole cell to minimize the effect of photobleaching during imaging.
476 We defined the accumulation time as the time when the fluorescence intensity at the damaged site
477 became greater than the fluorescence intensity at the non-damaged site by three times the standard
478 deviation (3xSD) for at least two consecutive time frames. We defined the dispersion time when
479 the fluorescence intensity at the damage site becomes less than that of the non-damaged site plus
480 3xSD for at least two consecutive time frames after the accumulation time. We defined the
481 retention time as the difference between the accumulation time and the dispersion time. We
482 measured at least three cells. The median values of accumulation times and retention times across
483 replicates for all repair proteins are listed in Data S2.
484
485 **Growth screening of repair protein knockout mutants**
486 Yeast cultures grown overnight in YPD at 25°C were diluted to OD600 = 0.1. The diluted
487 cultures were spotted onto YPD, YPD + 0.01% SDS, and YPD + 25 μg/ml CFW plates. After 3
488 days of incubation at 25°C or 37°C (Heat stress), the growth of the YPD plate and other plates
489 was compared. We performed the screening of two independent colonies. The strains were from
490 the yeast deletion collection library (62) except for *smi1*Δ, *las17*Δ, *skg6*Δ, *end3*Δ, *vrp1*Δ, and
491 *sla2*Δ. Only the mutants that showed sensitivity to the stress in two independent colonies were
492 defined as sensitive to the stress. The results of the screening are shown in Data S2.
493

494 **Spot assay**
495 Yeast cultures grown overnight in YPD at 25°C were diluted to OD600 = 0.1. The four-fold serial
496 dilutions of cultures were spotted onto the indicated plates.
497
498 **Immunoblotting**
499 Yeast cells in the early log phase (OD600 = 0.1-0.3) were treated with 0.02% SDS. 5 ml of yeast
500 cells were collected before SDS treatment, 1 hr after SDS treatment, and 2 hr after SDS treatment.
501 Cells were flash frozen by liquid nitrogen and stored at -80°C. Cells were resuspended in 120µl of
502 cold lysis buffer (0.25 M NaOH and 1% β-mercaptoethanol) and incubated on ice for 10 min. 20
503 µl of trichloroacetic acid was added to the lysates. After 10 min of incubation on ice, the lysates
504 were spun down, and the supernatant was discarded. The precipitates were washed with 500µl
505 ice-cold acetone twice and dried at room temperature. The precipitates are resuspended in SDS
506 polyacrylamide gel electrophoresis (PAGE) sample buffer [63 mM tris-Cl (pH 6.8), 2% SDS, 1%
507 β-mercaptoethanol, 0.01% Bromophenol Blue, and 10% glycerol]. Immunoblotting was
508 performed with anti-mini c-Myc (SANTA CRUZ, sc-40), anti-GFP (Roche Merck,
509 11814460001), or anti-tubulin α (Bio-Rad, MCA78G) antibodies.
510
511 **Statistical Analysis**
512 Dunnett's multiple comparison test, Welch's t-test, and Mann–Whitney U test were performed
513 with Python software (v. 3.11). Gene enrichment analysis and Fisher's exact test were performed
514 with R software (v. 4.2.2).
515
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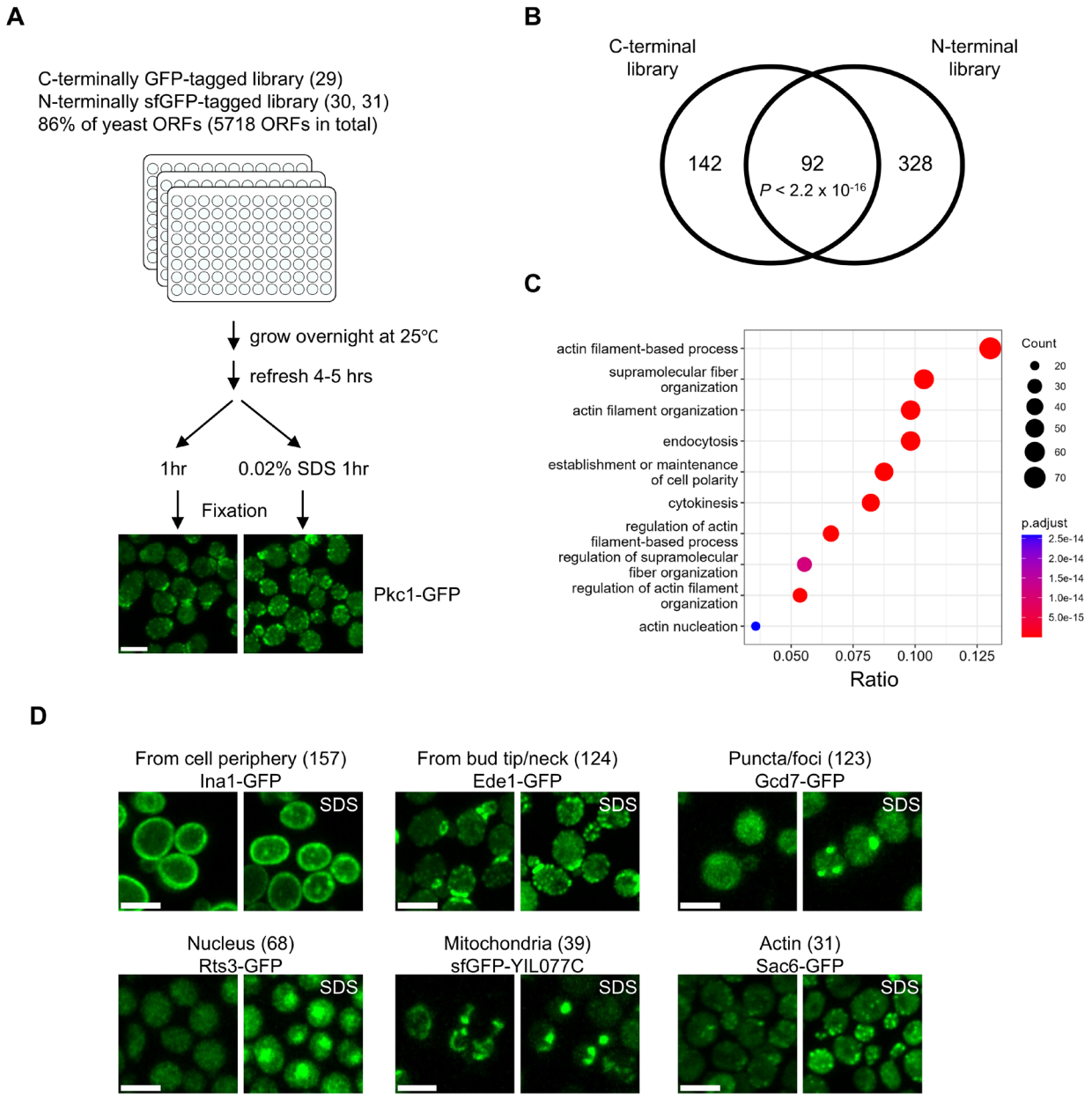

Fig. 1 Protein relocalization in response to SDS treatment.
(A) Schematic representation of screening methodology and images of Pkc1-GFP with or without 0.02 % SDS treatment are shown. Scale bar, 5 µm. **(B)** Overlap of screening hits of C- and N-terminal libraries. P value for the significance of the overlap is indicated. Fisher's exact test was performed. **(C)** GO analysis of biological processes for the screening hits. Successfully observed proteins were used as the background protein sets. **(D)** Screening hits for six relocalization classes and the images of representative proteins in each class were shown. Numbers in parentheses indicate the number of proteins in the class. Scale bar, 2 µm.

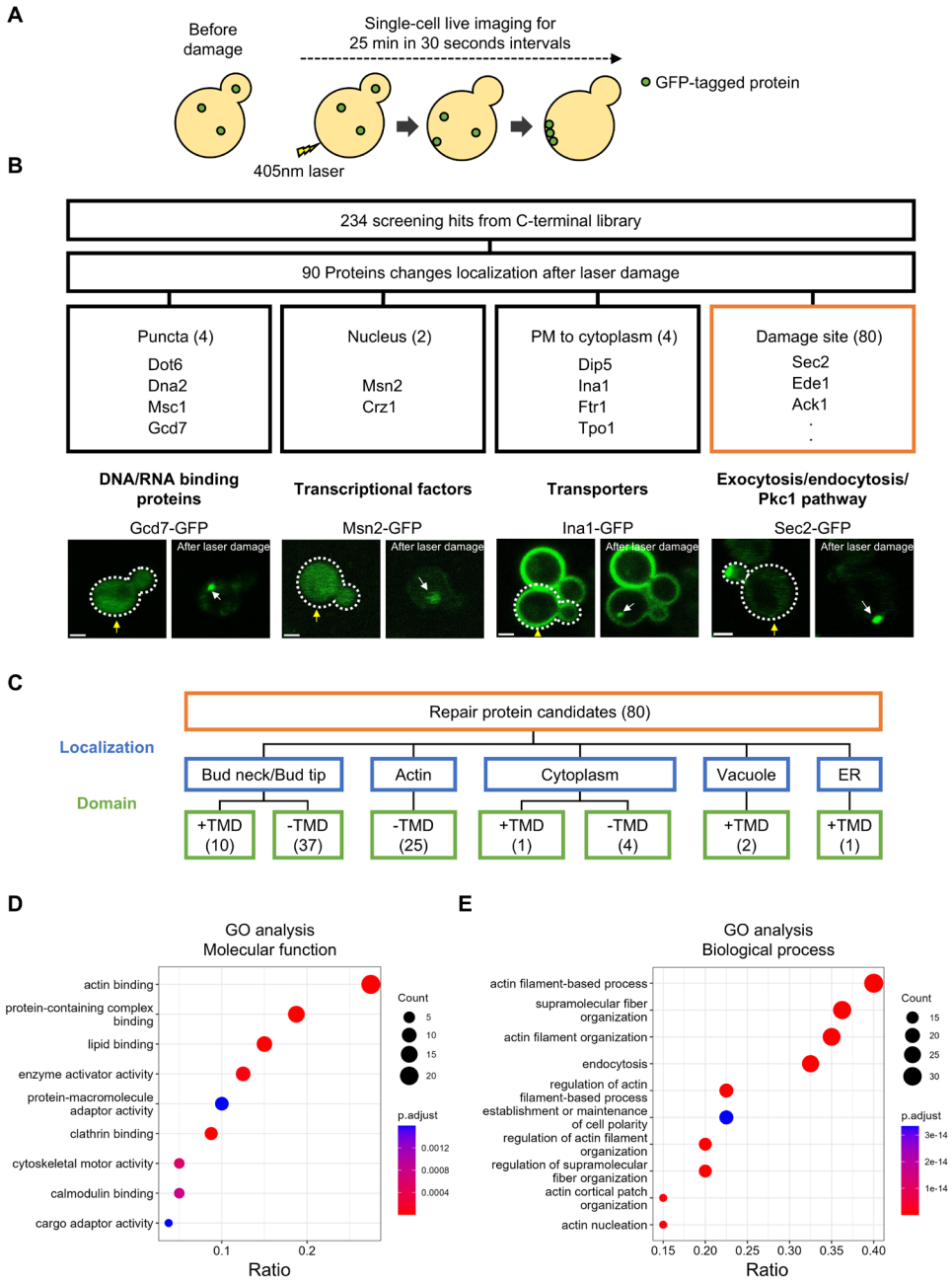

Fig.2 Laser damage assays identified 80 repair protein candidates.
(A) Schematic representation of screening methodology. Cells were imaged for 25 min in 30-second intervals after 405 nm laser damage. **(B)** 90 proteins change localization after laser damage. The classification of the repair protein candidates based on their localization changes, representative proteins, and representative biological processes in each category is shown. Scale bar, 2 µm. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. **(C)** Classification of repair protein candidates based on subcellular localization and domain. TMD+ represents the proteins that have transmembrane domains. The existence of transmembrane domains was predicted by TMHMM (63) **(D)** GO analysis of molecular functions for the repair protein candidates. The proteins whose localization changes in response to SDS treatment were used as background protein sets. **(E)** GO analysis of biological processes for the repair protein candidates. The proteins whose localization changes in response to SDS treatment were used as background protein sets. Ratio of the number of proteins associated with a specific GO term to the total number of proteins in the background is shown.

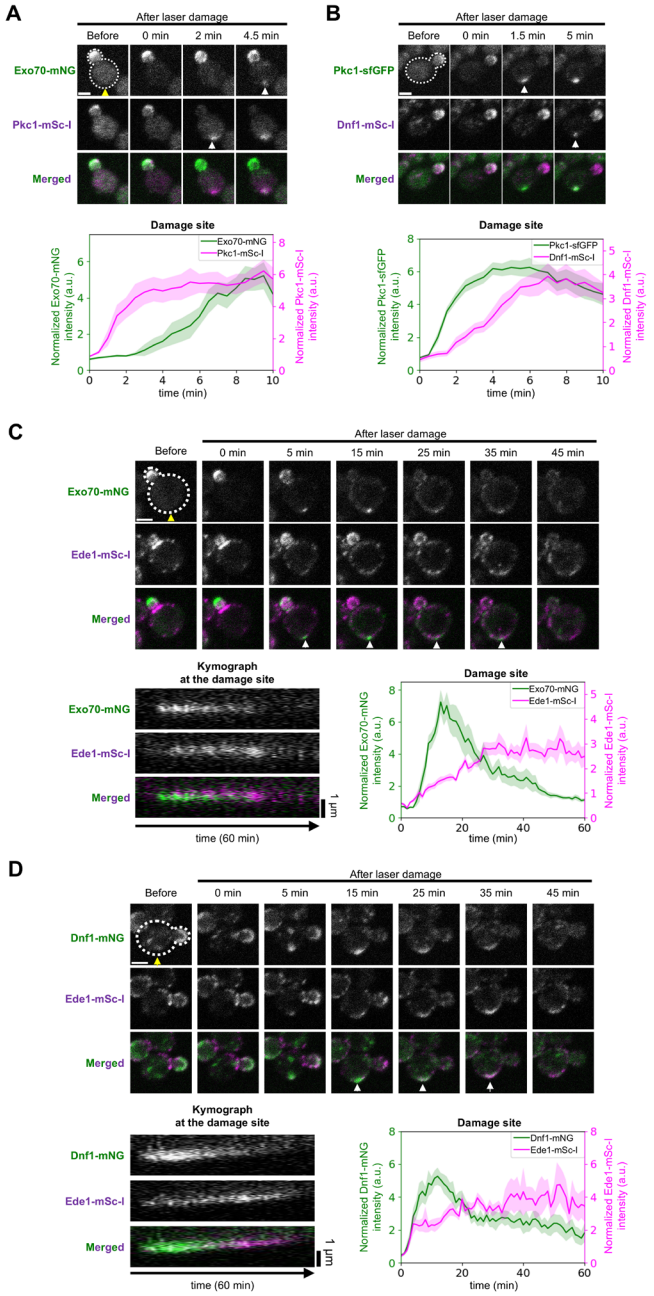

Fig. 3 The temporal order of the Pkc1 accumulation, polarized exocytosis, and CME at the damage site.

(A) The representative images and normalized fluorescence intensity of Exo70-mNG (Green) and Pkc1-mSc-I (Purple) at the damage site after laser damage. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. n = 10 cells. **(B)** The representative images and normalized fluorescence intensity of Pkc1-sfGFP (Green) and Dnf1-mSc-I (Purple) at the damage site after laser damage. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. n = 8 cells. **(C)** Representative images, kymograph at the damage site, and fluorescence intensity at the damage site of Exo70-mNG (Green) and Ede1-mSc-I (purple). n = 10 cells. Yellow arrows show the damage site. White arrows show the coaccumulation of Exo70-mNG and Ede1-mSc-I at the damage site. **(D)** Representative images, kymograph at the damage site, and fluorescence intensity at the damage site of Dnf1-mNG (Green) and Ede1-mSc-I (purple). n = 9 cells. Yellow arrows show the damage site. White arrows show the coaccumulation of Dnf1-mNG and Ede1-mSc-I. Lines and shaded regions are the mean and the standard error of the mean. White scale bar, 2 μm.

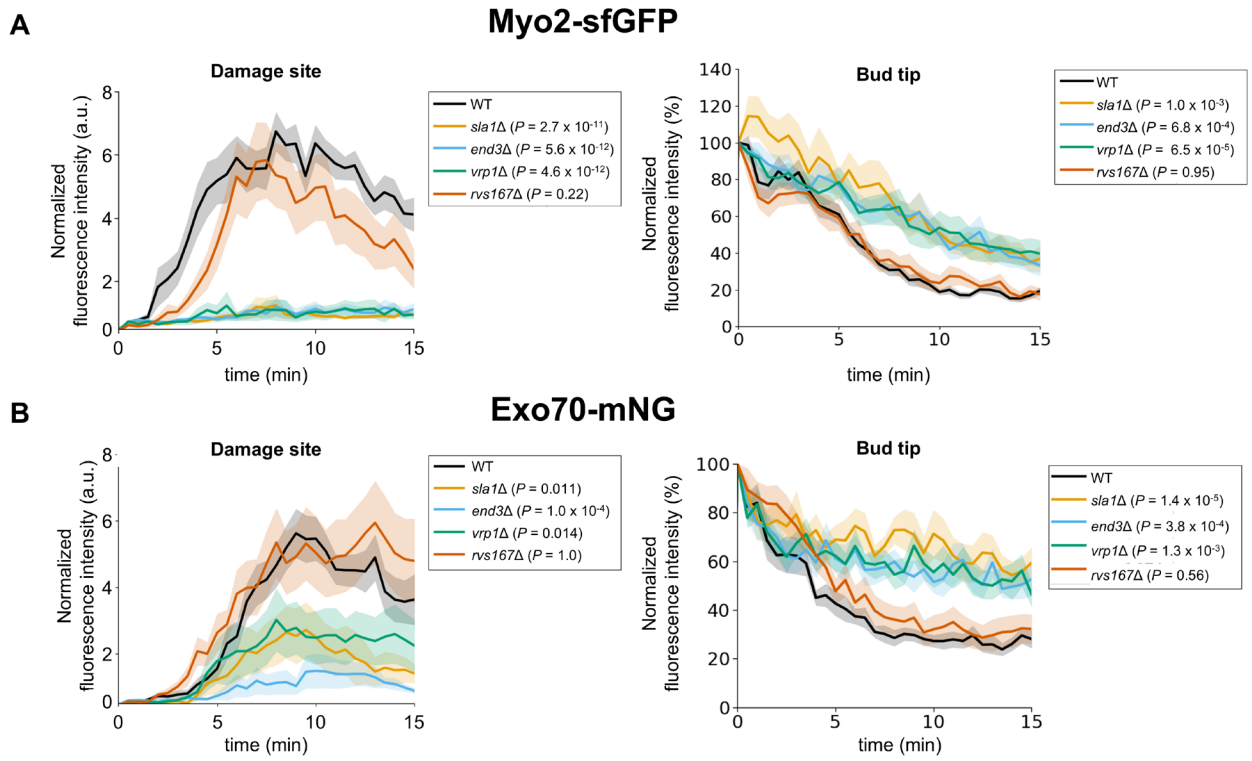

Fig. 4 CME proteins are required for polarized exocytosis at the damage site.
(A) The quantification results of fluorescence intensity of Myo2-sfGFP at the damage site and at the bud tip. n = 13 for WT, n = 12 for *end3*Δ, *sla1*Δ, *rvs167*Δ, and *vrp1*Δ. **(B)** The quantification results of the fluorescence intensity of Exo70-mNG at the damage site. n = 18 for WT, n = 12 for *end3*Δ, *sla1*Δ, and *rvs167*Δ, n = 14 for *vrp1*Δ. Lines and shaded regions are the mean and standard error of the mean. The maximum fluorescence intensity at the damage site or fluorescence intensity changes at the bud tip were compared between the WT and each CME mutant using Dunnett's multiple comparison test.

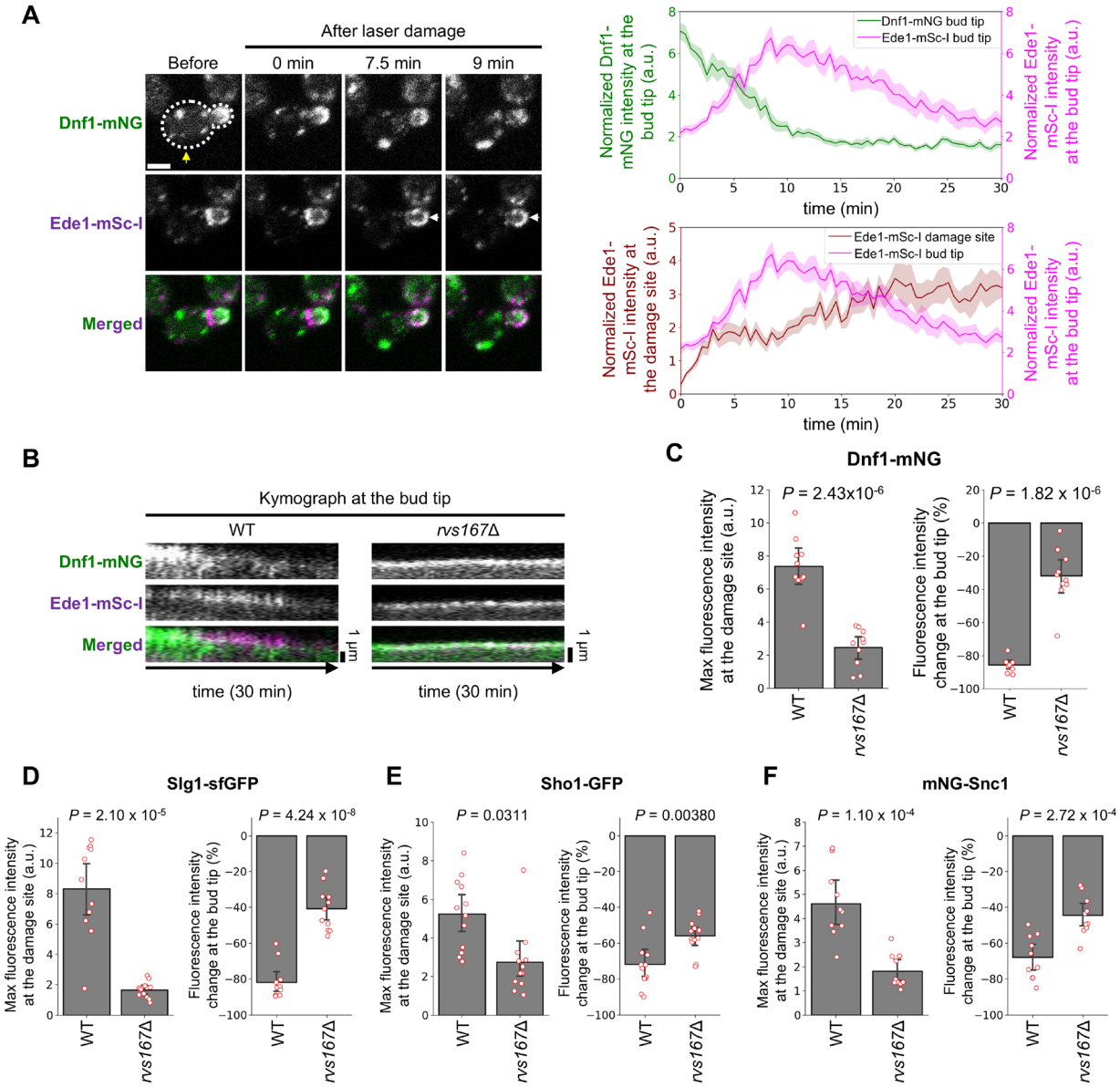

Fig. 5 CME at the bud tip directs repair proteins with TMDs to the damage site. **(A)** Representative images and the normalized fluorescence intensity at the bud tip of Dnf1-mNG and Ede1-mSc-I. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. Scale bar, 2 μm. **(B)** Kymograph of Dnf1-mNG and Ede1-mSc-I at the bud tip in WT and *rvs167*Δ. n = 10 cells. **(C)-(F)** Max fluorescence intensity at the damage site and fluorescence intensity changes at the bud tip in WT and *rvs167*Δ. n = 10 cells for Dnf1-mNG. n = 11 cells for Slg1-sfGFP. n = 12 cells for Sho1-GFP. n = 10 cells for mNG-Snc1. Welch's t-test was performed.

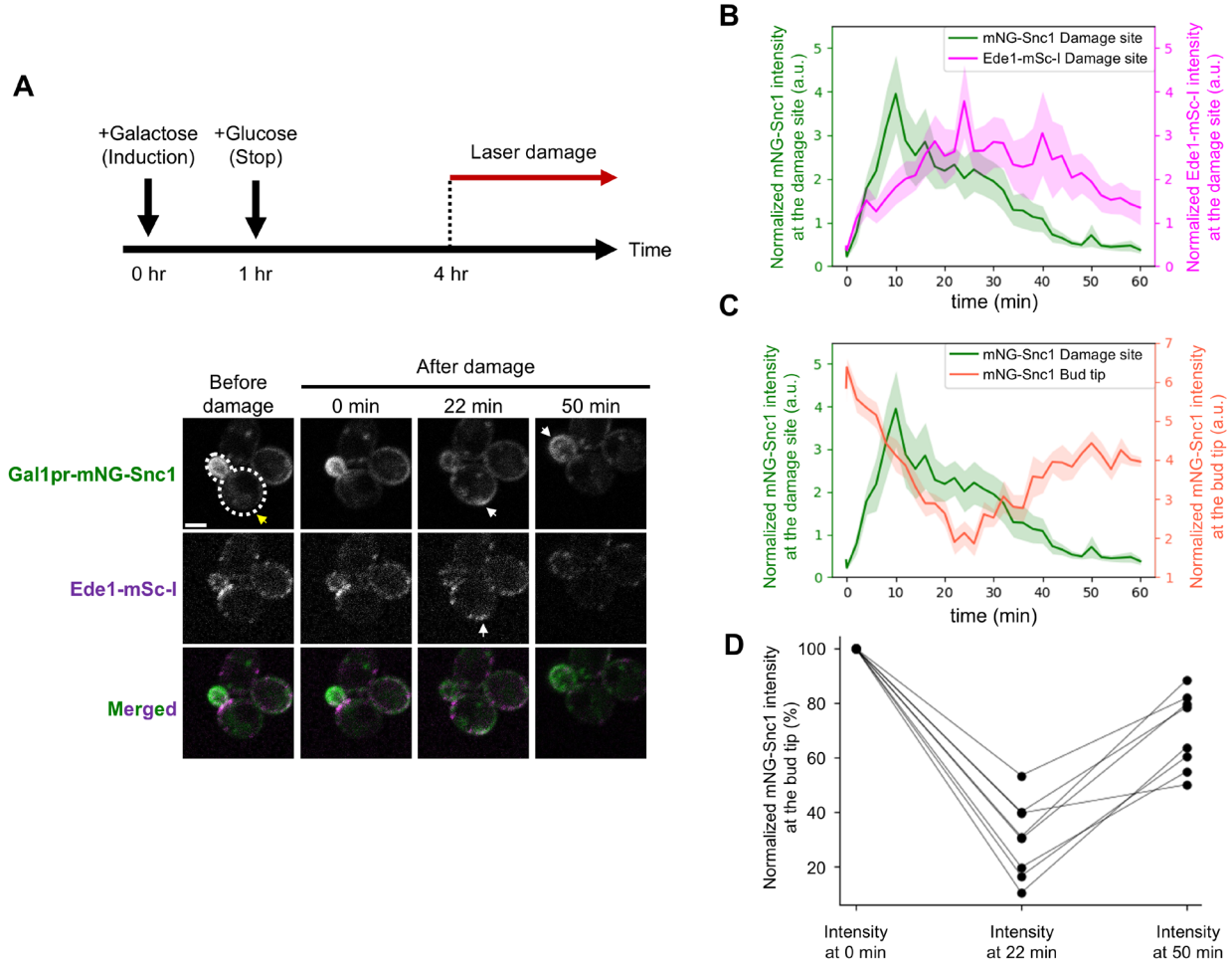

Fig. 6 mNG-Snc1 is recovered from the damage site to the bud tip after PM repair. **(A)** Schematic of transient expression induction of mNG-Snc1 by Gal1 promoter and representative images of mNG-Snc1 and Ede1-mSc-I. After transcription activation of the Gal1 promoter by adding 3% galactose, we stop the expression by transferring the cells to glucose media. The cells were incubated for at least 3 hours before the laser damage assay. Yellow arrows show the damage site. White arrows showed the recruitment of fluorescence signals. Scale bar, 2 µm. **(B)** Quantification of mNG-Snc1 (Green) and Ede1-mSc-I (purple) at the damage site. **(C)** Quantification of mNG-Snc1 at the bud tip (Green) and at the damage site (tomato). **(D)** The changes in the normalized mNG-Snc1 signal at the bud tip. n = 8 cells.

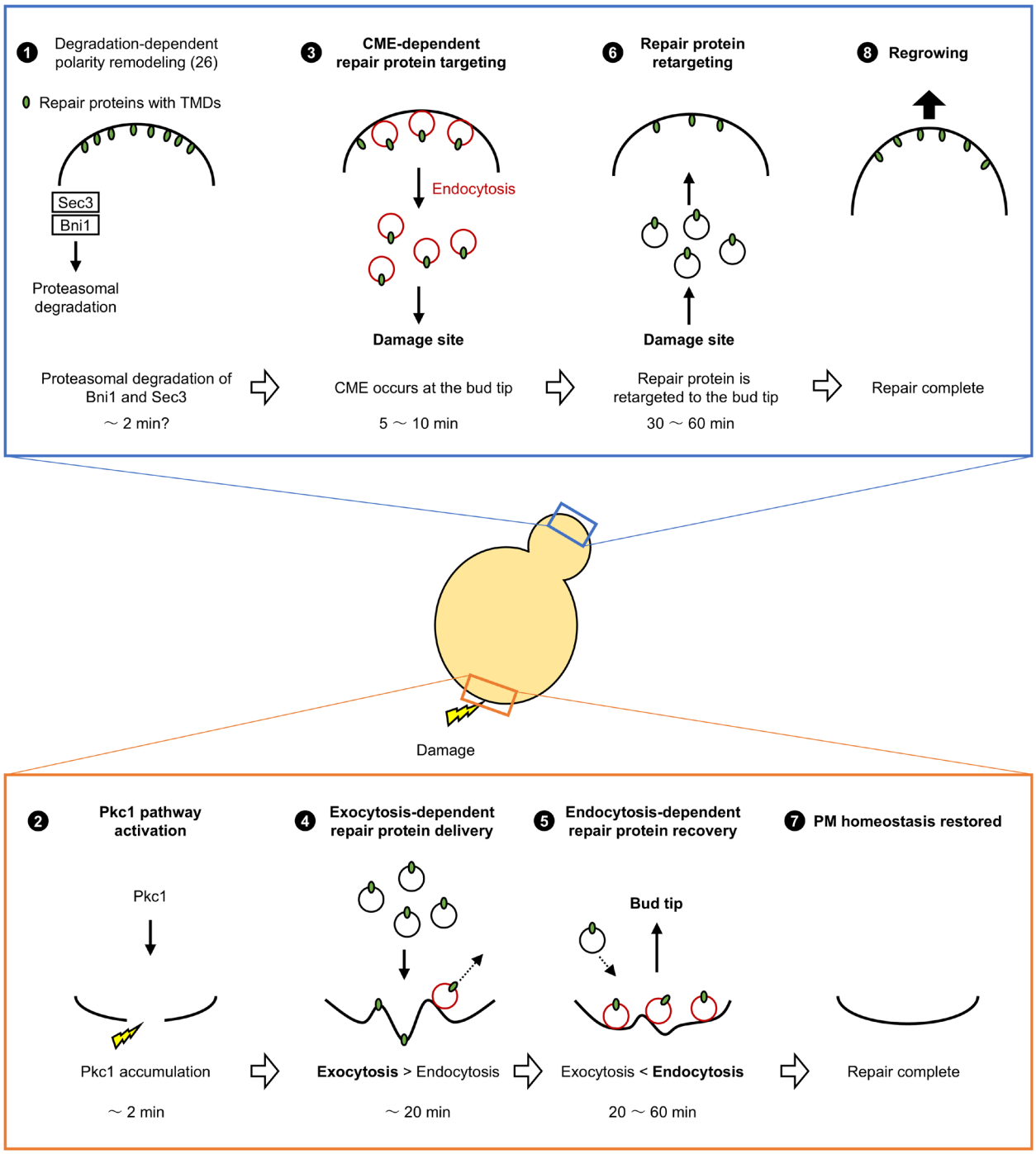

Fig. 7 Model of spatiotemporal cellular responses to PM damage in budding yeast.
We showed the hypothetical model of spatiotemporal PM damage responses in budding yeast. First, the degradation of Sec3 and Bni1 resolved the polarity competition between the bud tip and the damage site (26). Within 10 min after laser damage, CME directs repair proteins with TMDs to the damage site from the bud tip. At the damage site, polarized exocytosis and CME simultaneously occur, with exocytosis predominating approximately within 20 min and with CME predominating approximately 20 min after laser damage. CME targets repair proteins with TMDs from the bud tip to the damage site. The endocytosed PM proteins are retargeted to the bud tip again after the PM repair is finished. The retargeted PM proteins may be involved in the resumption of cell growth after PM repair. The numbers represent the temporal order of events.

Supplementary Materials for

Large-scale identification of plasma membrane repair proteins revealed spatiotemporal cellular responses to plasma membrane damage

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This PDF file includes:
Figs. S1 to S10

Other Supplementary Materials for this manuscript include the following:
Data S1 and S2

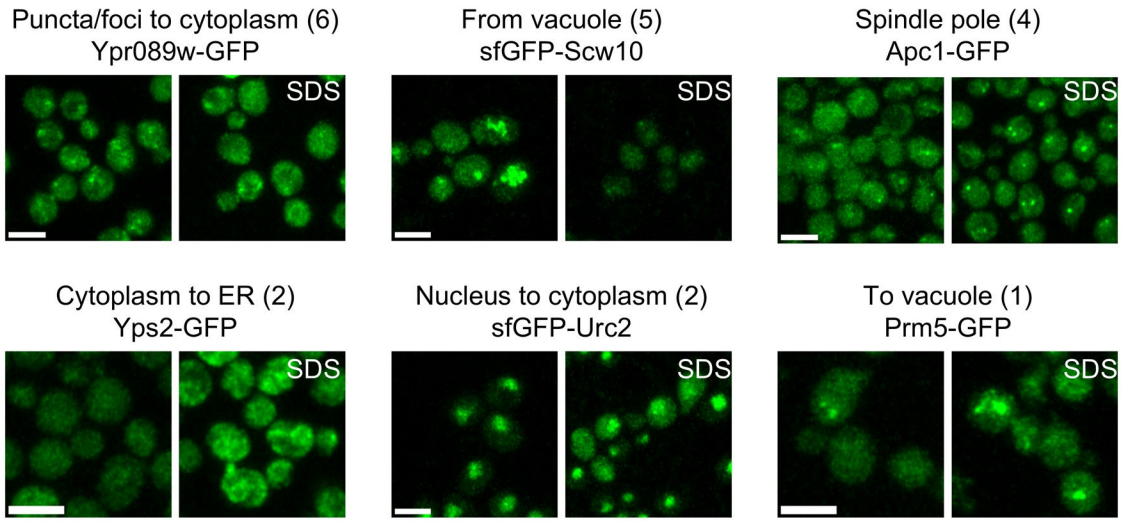

Fig. S1 Minor classes of localization changes in response to SDS treatment.
Representative images of GFP- or sfGFP-tagged proteins in minor classes with or without 0.02% SDS treatment are shown. Numbers in parentheses indicate the number of proteins in the class. Scale bar 2 μm.

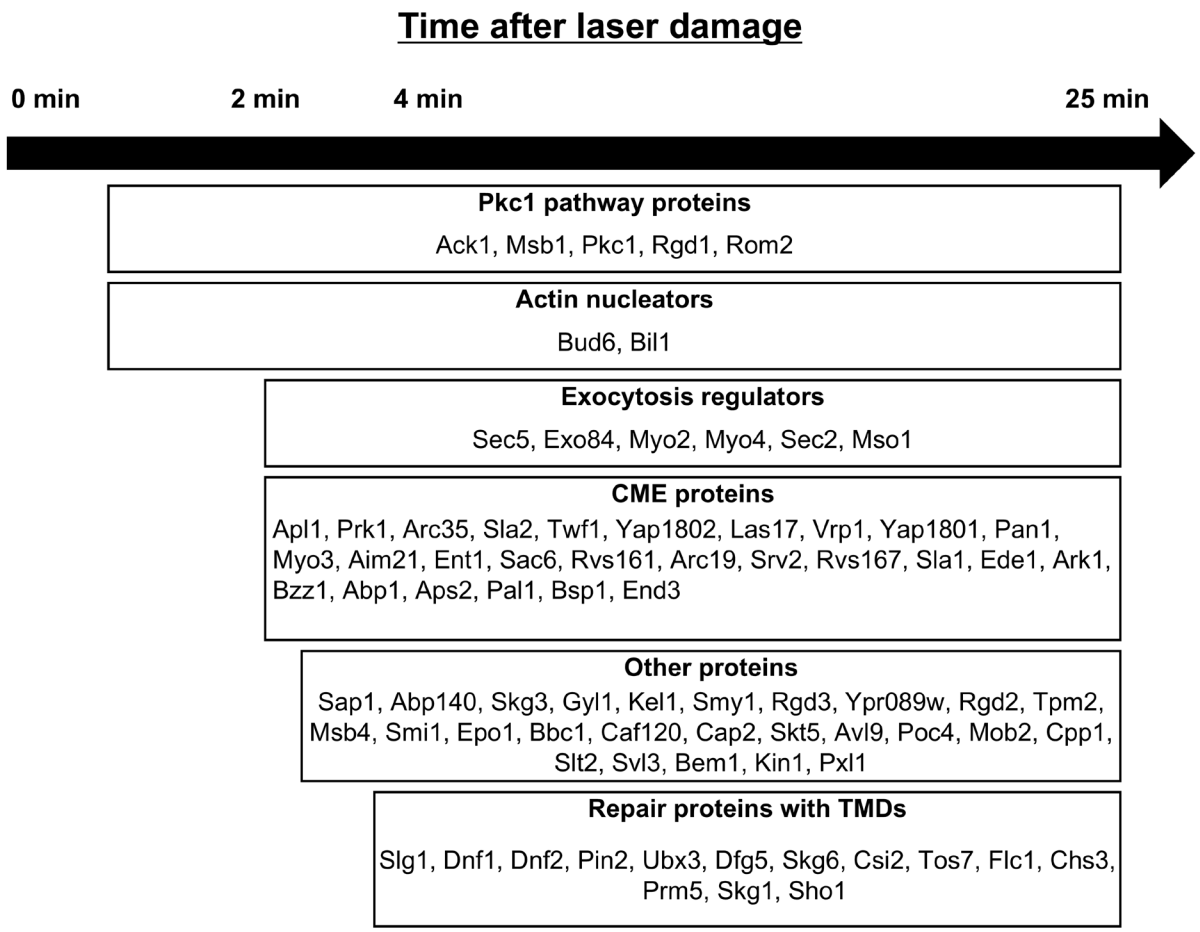

Fig. S2 Summary of the accumulation time of repair proteins.
The repair protein candidates were grouped based on their functions. The box ranges from the accumulation times to the dispersion times of grouped proteins. See also Data S2.

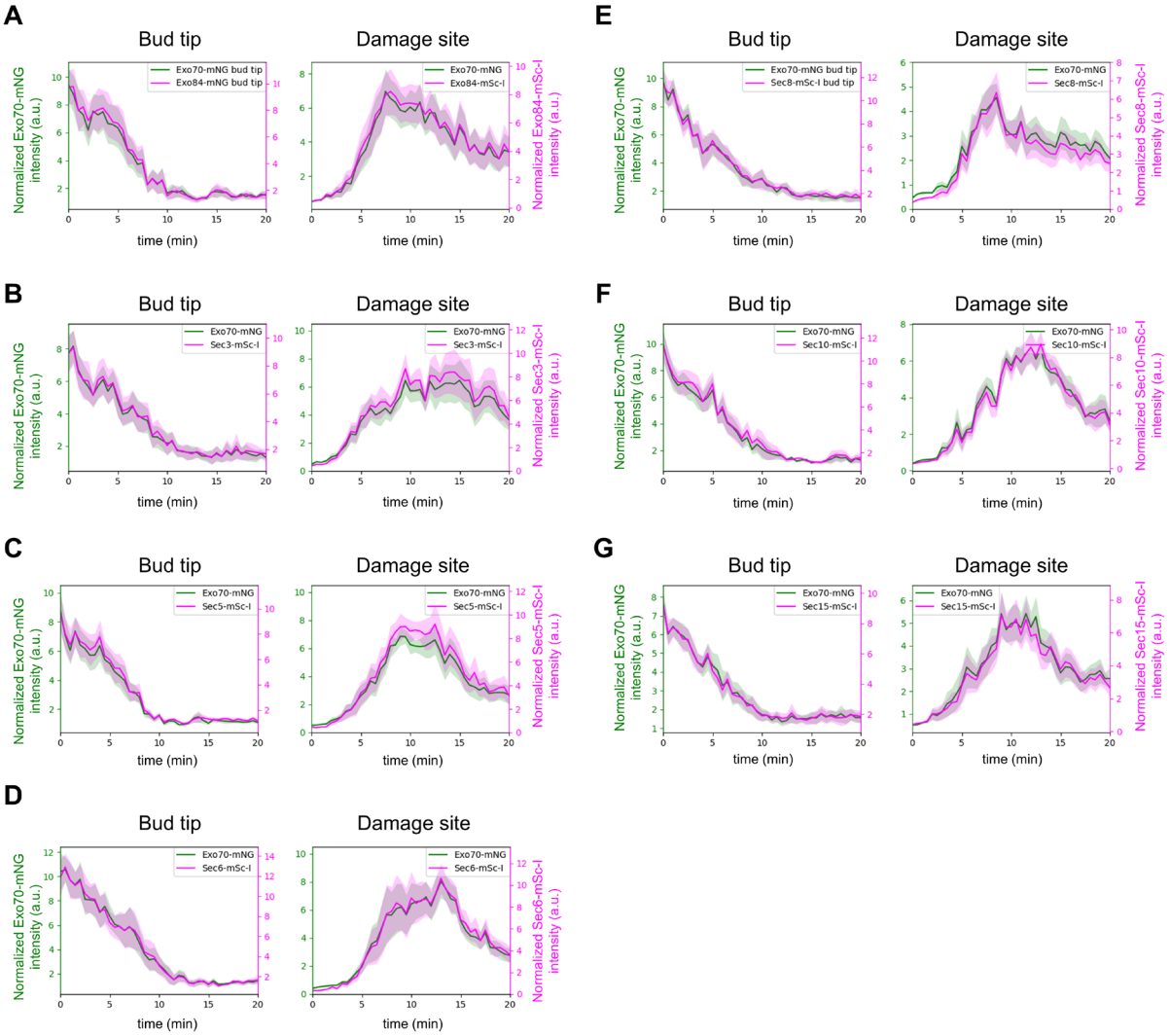

Fig. S3 Accumulation patterns of exocyst subunits.
Fluorescence intensity at the damage site of Exo70-mNG (Green), **(A)** Exo84-mSc-I, **(B)** Sec3-mSc-I, **(C)** Sec5-mSc-I, **(D)** Sec6-mSc-I, **(E)** Sec8-mSc-I**, (F)** Sec10-mSc-I**, and (G)** Sec15-mSc-I (purple). Lines and shaded regions are the mean and the standard error of the mean. n = 10 cells.

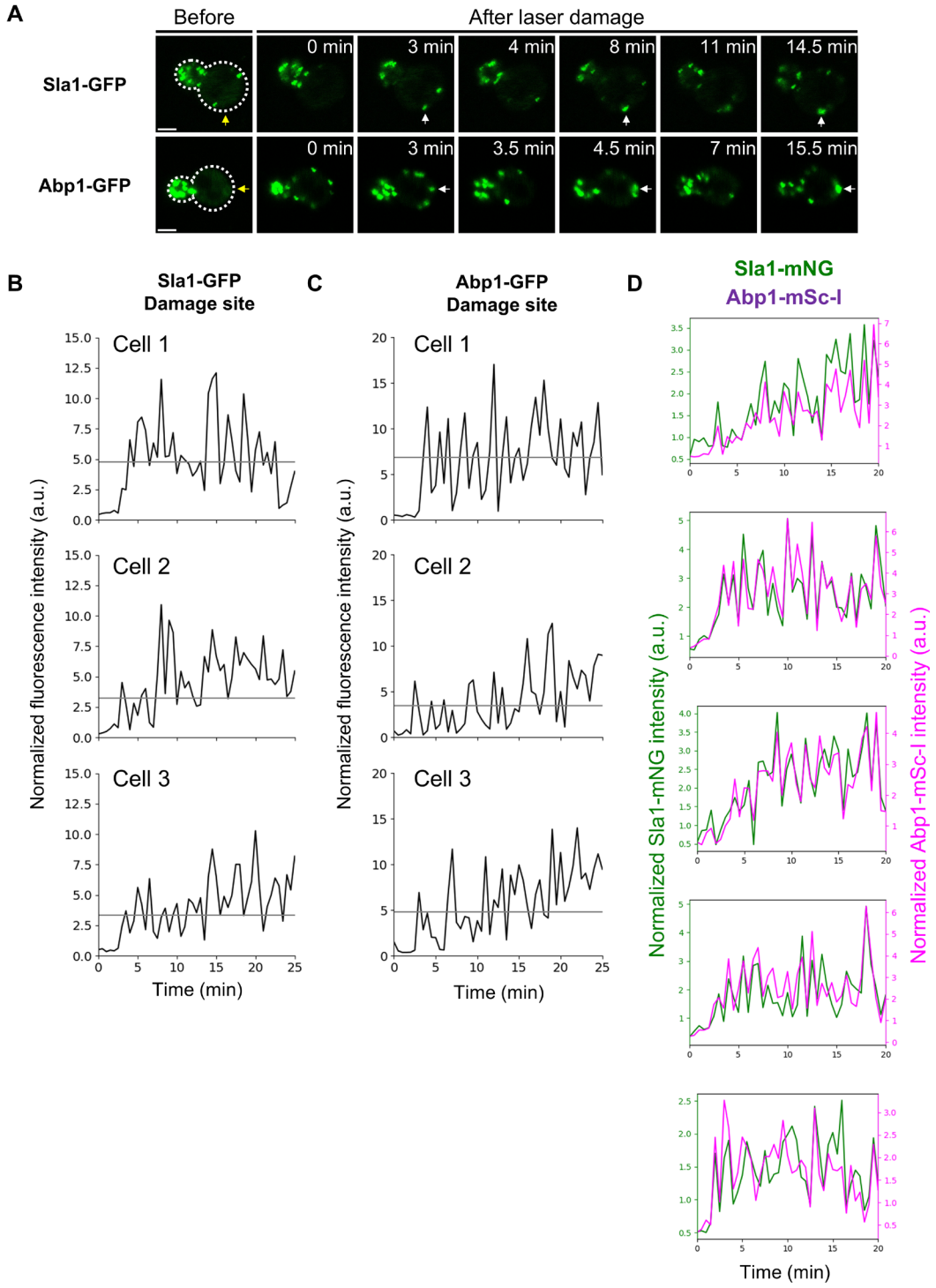

Fig. S4 CME continuously occurs at the damage site.
(A) Representative images of Sla1-GFP and Abp1-GFP. Yellow arrows show the damage site. White arrows showed the recruitment of fluorescence signals. **(B)(C)** Quantification of Sla1-GFP and Abp1-GFP signal at the damage site for three cells. Gray: the fluorescence intensity at the non-damaged site (control) + three times the standard deviation. **(D)** Fluorescence intensity at the damage site of Sla1-mNG (Green) and Abp1-mSc-I (magenta) is shown. n = 5 cells.

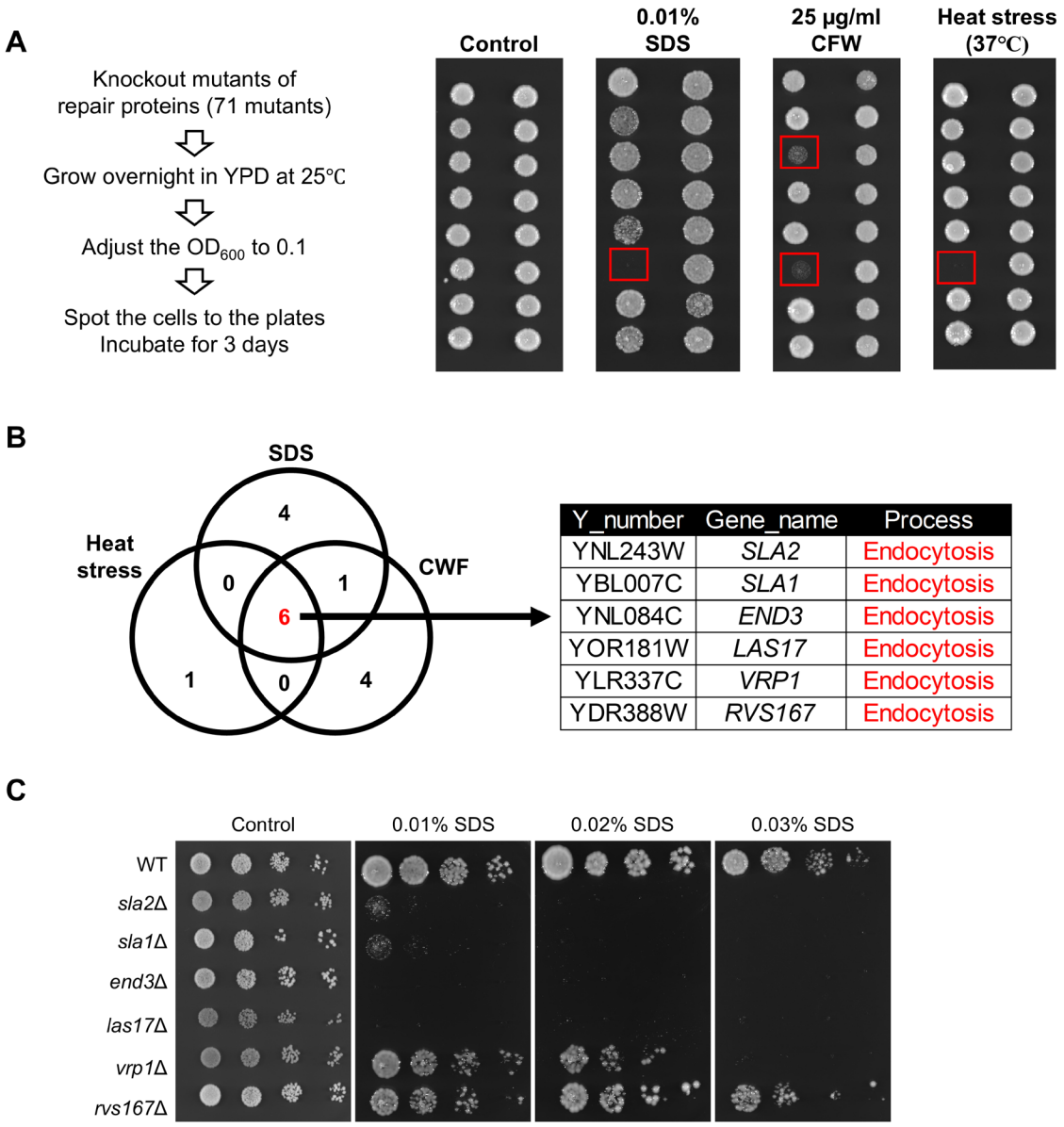

Fig. S5 Growth screening of repair protein knockout mutants in PM/cell wall damage conditions.
(A) Schematic workflow and representative results of growth screening. The red square shows the sensitive mutants. Only the mutants that showed sensitivity to the stresses in two independent colonies were defined as sensitive to the stress. The results of the screening are shown in Data S2. **(B)** The overlap of the screening hits in the three PM/cell wall damage conditions is shown. **(C)** Growth assay of CME mutants. A four-fold serial dilution of the yeast culture was prepared and spotted.

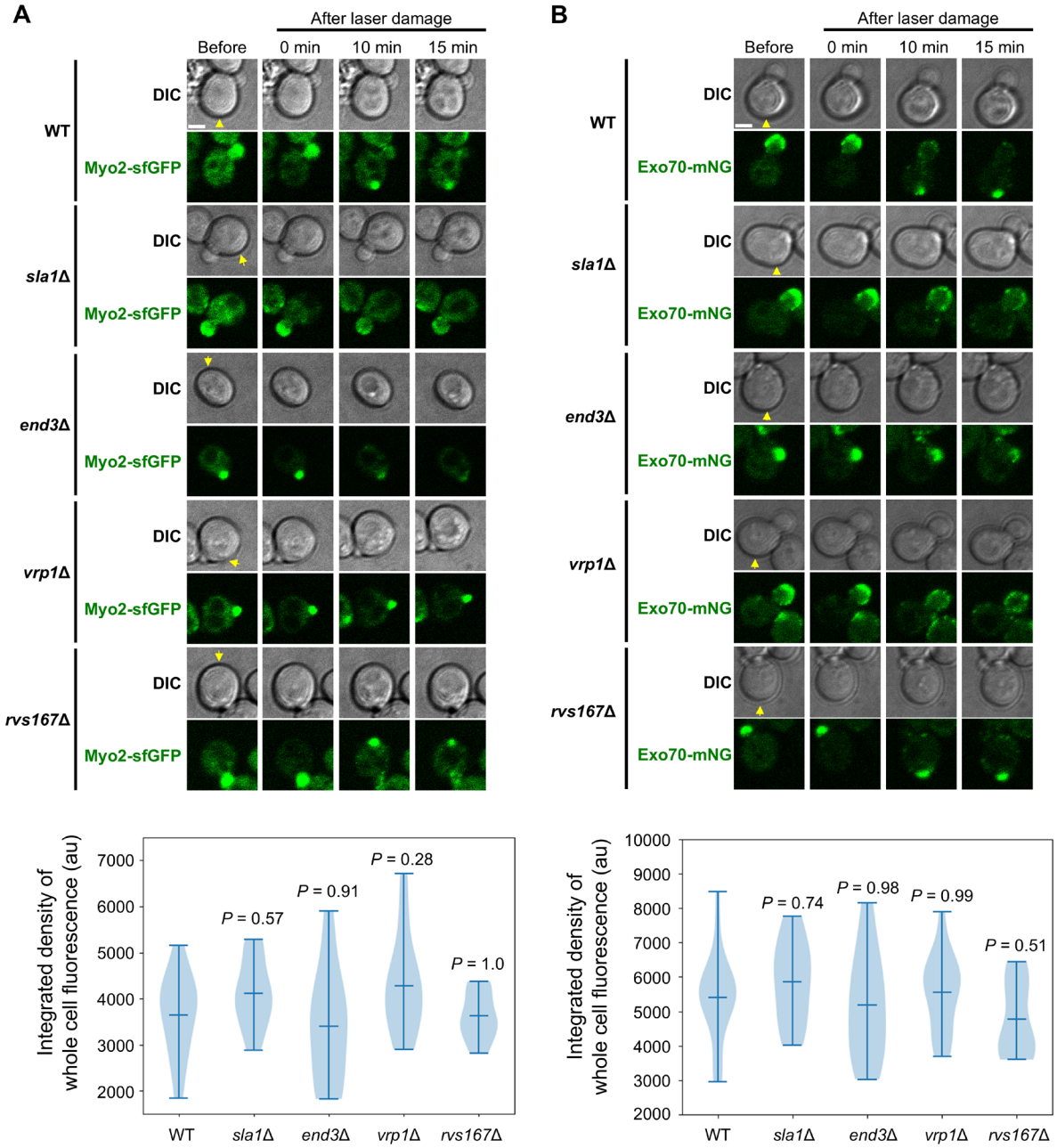

Fig. S6 Representative images of Myo2-sfGFP and Exo70-mNG in CME mutants.
(A) Representative images and whole cell fluorescence quantification of Myo2-sfGFP in the indicated strains. The integrated density of whole cell fluorescence of Myo2-sfGFP before laser damage is compared between WT and CME mutants using Dunnett's test. n = 13 for WT, n = 12 for *end3*Δ, *sla1*Δ, *rvs167*Δ, and *vrp1*Δ. Scale bar 2 µm. **(B)** Representative images and whole cell fluorescence quantification of Exo70-mNG in the indicated strains. The integrated density of whole cell fluorescence of Exo70-mNG before laser damage is compared between WT and CME mutants using Dunnett's test. n = 18 for WT, n = 12 for *end3*Δ, *sla1*Δ, and *rvs167*Δ, n = 14 for *vrp1*Δ. Scale bar 2 µm. Yellow arrows show the damage site.

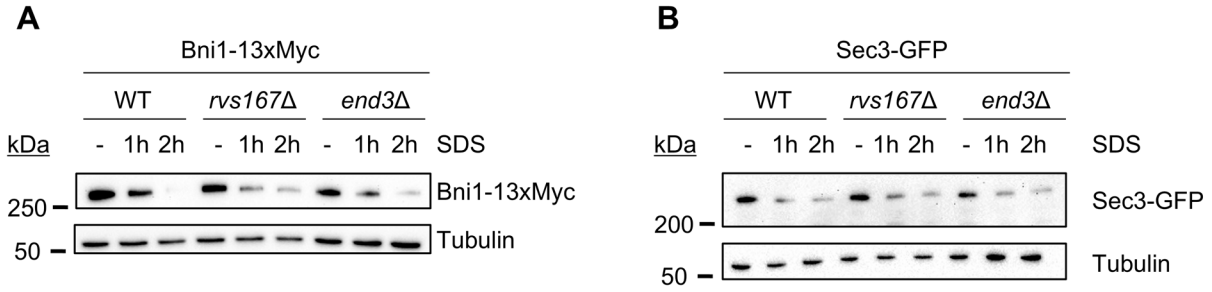

Fig. S7 CME proteins are not required for the degradation of Bni1-13xMyc and Sec3-GFP after SDS treatment.

(A) Immunoblotting of Bni1-13xMyc before and after SDS treatment (Before (-), 1hr, and 2hr) in WT, *rvs167*Δ, and *end3*Δ. **(B)** Immunoblotting of Sec3-GFP before and after SDS treatment (Before (-), 1hr, and 2hr) in WT, *rvs167*Δ, and *end3*Δ.

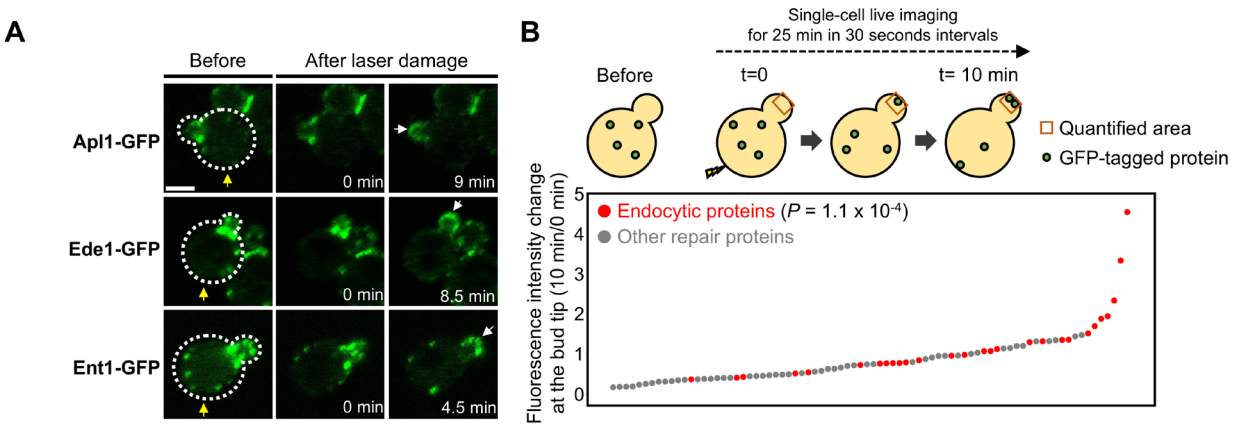

Fig. S8 Fluorescence intensity changes of CME proteins at the bud tip.
(A) Representative images of Apl1-GFP, Ede1-GFP, and Ent1-GFP. **(B)** The ratio of normalized fluorescence intensity at 10 min at the bud tip to the normalized fluorescence intensity at 0 min at the bud tip for all repair protein candidates. Red: CME proteins. The list of CME proteins is adapted from (53). The *P* value was calculated by the two-sided Mann–Whitney U test.

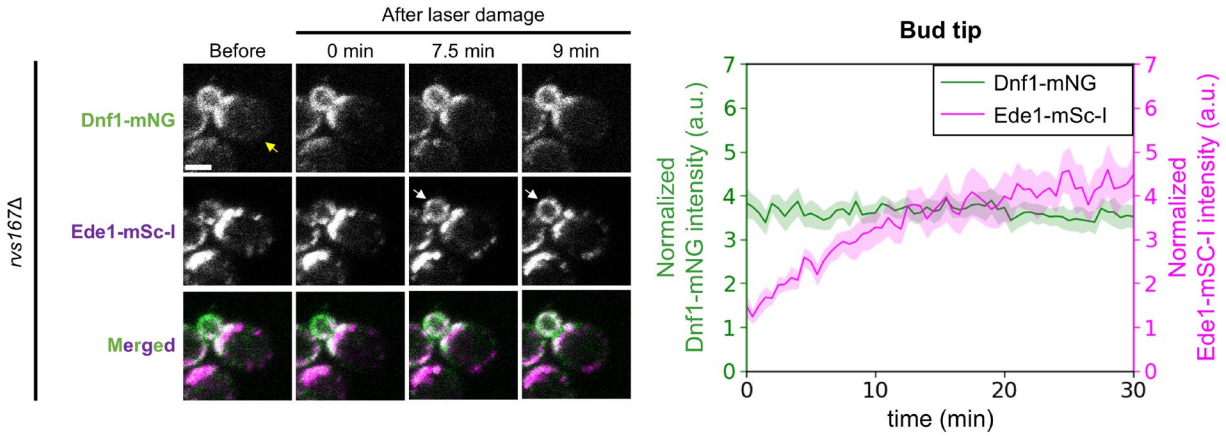

Fig. S9 Time course fluorescence intensity changes of Dnf1-mNG and Ede1-mSc-I at the bud tip in *rvs167*Δ.

Representative images of Dnf1-mNG and Ede1-mSc-I in *rvs167*Δ and normalized fluorescence intensity of Dnf1-mNG (Green) and Ede1-mSc-I (Purple) at the bud tip in *rvs167*Δ. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. Scale bar 2 µm.

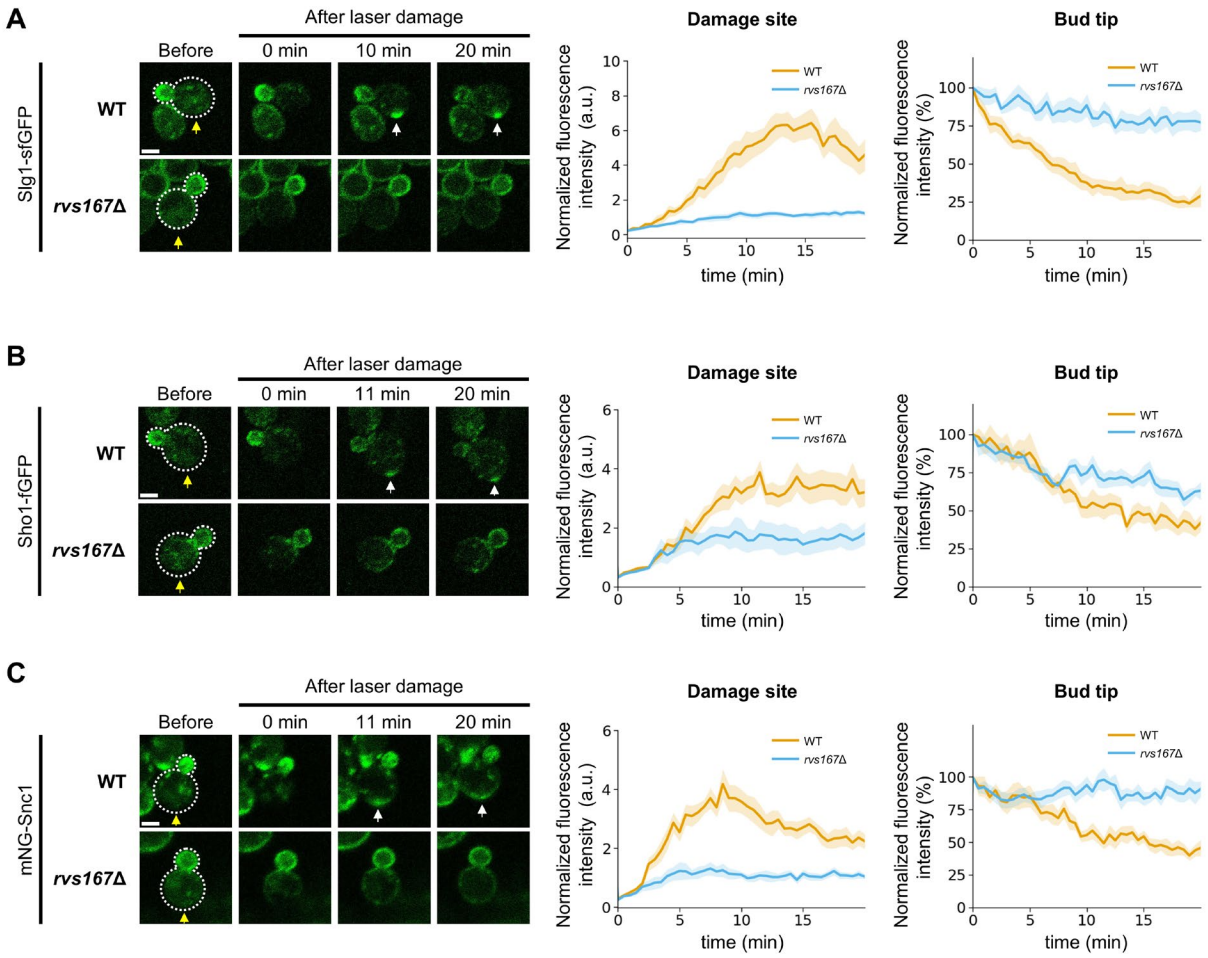

Fig. S10 Time course fluorescence intensity changes of repair proteins with TMDs.
(A) Representative images of Slg1-sfGFP and the normalized fluorescence intensity at the damage site and at the bud tip in WT and *rvs167*Δ. Scale bar 2 µm. **(B)** Representative images of Sho1-GFP and the normalized fluorescence intensity at the damage site and at the bud tip in WT and *rvs167*Δ. Scale bar 2 µm. **(C)** Representative images of mNG-Snc1 and the normalized fluorescence intensity at the damage site and at the bud tip in WT and *rvs167*Δ. Scale bar 2 µm. Yellow arrows show the damage site. White arrows show the recruitment of fluorescence signals. Scale bar 2 µm.

Data S1. (separate file) Proteins whose localization changes in response to the SDS treatment.

Data S2. (separate file) Repair protein candidates.