

1 **Force-dependent remodeling of a tight junction protein ZO-1 is regulated by phase**
2 **separation**

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26

27 **Summary**

28 Although the physiological importance of biomolecular condensates is widely recognized,
29 how it is controlled in time and space during development is largely unknown. Here we
30 show that a tight junction protein ZO-1 forms cytoplasmic condensates in the
31 trophectoderm (TE) of the mouse embryo before E4.0. These disappear via dissolution,
32 and ZO-1 accumulates at the cell junction as the blastocyst cavity grows, and internal
33 pressure on TE cells increases. In contrast, the dissolution is less evident in TE cells
34 attached to the inner cell mass, as they receive weaker tensile forces. Furthermore,
35 analyses using MDCK cells have shown that the ZO-1 condensates are generated and
36 maintained by liquid-liquid phase separation. Our study also highlights that the dynamics
37 of these condensates depends on the physical environment via the interaction between
38 ZO-1 and F-actin. We propose that the force-dependent regulation of ZO-1 condensation
39 contributes to establishing robust cell-cell adhesion during early development.

40

41 **Keywords**

42 Cell-to-cell adhesion, ZO-1, tight junction, mechanical force, mouse development,
43 condensate, liquid-liquid phase separation (LLPS), intrinsically disordered region (IDR).

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45

46 **Introduction**

47 Mechanical forces are generated at different times and in different tissues during the
48 development of organisms, due to the dynamic movement and shape change of cells and
49 tissues (Bodor et al., 2020), growth of a cell mass by proliferation (Godard and
50 Heisenberg, 2019), the removal of cells by apoptosis (Teng et al., 2017), luminal pressure
51 (Chan et al., 2019), and shear stress of body fluids such as blood (Paolini and Abdelilah-
52 Seyfried, 2018), etc. All of these forces can contribute to the morphogenesis of organs
53 through mechanochemical feedback mechanisms (Hannezo and Heisenberg, 2019).
54 Although the presence and physiological importance of such forces have long been
55 implicated in the homeostasis as well as morphogenesis of living organisms (Hallou and
56 Brunet, 2020; Thompson, 1917), how cells sense and respond to these forces are not fully
57 understood at the molecular level. In this study, we demonstrate that the remodeling of
58 ZO-1 through the regulation of liquid-liquid phase separation (LLPS) is mechanically
59 controlled.

60 In our previous study using *Xenopus laevis* embryos, we demonstrated that among a
61 number of identified phosphoproteins, cell junction- as well as focal adhesion-related
62 proteins are highly phosphorylated immediately after the application of mechanical forces
63 by centrifugation or compression (Hashimoto et al., 2019). Intriguingly, a global analysis
64 of the phosphoproteome suggested that the mechanical stimuli induced the embryonic
65 cells to adopt an epithelial state rather than a mesenchymal state, known as the
66 mesenchymal-epithelial transition (MET), which is opposite to the epithelial-
67 mesenchymal transition (EMT), a well-characterized phenomenon in some
68 developmental contexts and in cancer pathogenesis in which cells compromise their cell-
69 to-cell adhesiveness (Baum et al., 2008). In fact, we confirmed that adherens- and tight
70 junction, revealed by the accumulation of C-cadherin (Choi et al., 1990; Ginsberg et al.,
71 1991) and ZO-1 (Anderson et al., 1988), respectively, became enhanced after the
72 application of force. We also found that F-actin, which is normally localized in the basal
73 and lateral domain of cells, accumulated at the apical domain, reinforcing the stiffness of
74 the cell cortex (Hashimoto et al., 2019). These observations suggest that these junctions
75 were remodeled following the force stimuli and that the cells adopted more epithelialized
76 states. This led us to propose that force-induced epithelialization is an important cellular
77 response to physical forces to maintain the integrity of a tissue, allowing it to acquire
78 robustness against forces which might perturb normal morphogenesis.

79 More recently, we showed that FGFR1 is activated, and that Erk2 is consequently
80 phosphorylated and translocated into the nucleus in ectodermal cells (Kinoshita et al.,
81 2020) to achieve MET during gastrulation. During the course of that study, we repeatedly
82 observed that ZO-1 condensates were found as puncta in ectoderm cells prior to, but not
83 after, gastrulation (Kinoshita et al., 2020). Based on previous reports that showed that
84 ZO-1 can undergo LLPS in zebrafish embryos (Schwayer et al., 2019) and cultured cells
85 (Beutel et al., 2019), we speculated that the ZO-1 LLPS is mechanically regulated,
86 particularly by dynamic morphogenesis known as epiboly during gastrulation which
87 imposes a tensile force on the ectoderm (Hernandez-Vega et al., 2017).

88 To extend the above study that employed *Xenopus laevis*, and to examine whether our
89 working model could be extrapolated to other species such as mammals, we focused on
90 mouse embryogenesis in this study. Basically, we obtained similar results to those with
91 *Xenopus* and found that cytoplasmic condensates of ZO-1 exist in the trophectoderm (TE)
92 cells of early mouse embryos before E4.0, although the condensate disappears upon
93 stretching of TE cells due to the expansion of the blastocyst cavity (blastocoel).

94 To confirm that ZO-1 condensates are the product of cellular LLPS, a physical process to
95 facilitate the demixing of proteins through protein condensation (Brangwynne, 2013), we
96 used on cultured cells in which condensates of GFP-ZO-1 were observed in various
97 conditions. Following both the treatment of a cell-permeabilizing agent digitonin (Shiina,
98 2019), and analyses with fluorescent recovery after photobleaching (FRAP), ZO-1
99 condensates have been shown to have typical properties of liquid droplets generated by
100 LLPS. We also identified that the N-terminal fragment of ZO-1, which contains
101 intrinsically disordered regions (IDRs) (Kato et al., 2012; Li et al., 2012), an unstructured
102 stretch of an amino acid sequence of low complexity, are sufficient for phase separation
103 of ZO-1. Finally, we highlighted the importance of cytoskeletal actin in the regulation of
104 ZO-1 assembly and propose that ZO-1 protein became liberated from the droplets and
105 was deployed to enhance the tight junction in a force-dependent manner. This was
106 supported by observations of MDCK cells in different culture conditions, including
107 wound healing.

108 The present study, which used early mouse embryos and cultured cells, reveals a
109 previously unknown role of physical force that regulates the condensation of ZO-1 and
110 contributes to the enhancement of cell-to-cell adhesion during early development.

111

112 **Results**

113 ***ZO-1 condensates in early mouse embryos***

114 To examine whether the behavior of ZO-1 protein in *Xenopus laevis* embryos which we
115 reported previously (Kinoshita et al., 2020) is conserved across species, especially in
116 mammals, we first performed immunostaining of ZO-1. We focused on E3.5 and E4.5
117 embryos since they hatch out of the zona pellucida (ZP) and expand their shape (Figure
118 1A). We fixed E3.5 and E4.5 embryos with paraformaldehyde and immunostained with a
119 ZO-1 antibody (Figure 1B). We found that the cells of E3.5 embryos showed a
120 significantly higher number of ZO-1 puncta in the cytoplasm relative to E4.5 embryos.
121 As development proceeded, the surface area of TE cells, particularly on the mural side,
122 expanded (Figure 1E) and became thinner (data not shown). We found that the number of
123 cytoplasmic ZO-1 puncta were reduced and ZO-1 signal intensity at the plasma
124 membrane in E4.5 embryos became much higher than those of E3.5 embryos at the
125 expense of its cytoplasmic pool (Figure 1C, D mural), without changing the total
126 fluorescence intensity of the protein (Figure 1F left). Importantly, this change coincides
127 well with the accumulation of F-actin at the cell cortex in E4.5 embryos (Figure 1B and
128 1F right). This result suggests that the shuttling of ZO-1 protein from the cytoplasmic
129 puncta to cell junctions occurs as development progressed. We observed a similar
130 behavior of ZO-1 protein using ZO-1-EGFP-expressing mouse embryos by live-imaging
131 (Katsunuma et al., 2016) (Video S1 and Figure S1).

132 Interestingly, however, in the polar TE cells attached to the inner cell mass (ICM) of
133 E4.5 embryos, a significant number of condensates remained in the cytoplasm (lower
134 panels in Figure 1B lower and Figure 1D). Consistently, the fluorescence intensity of anti-
135 ZO-1 immunostaining at the plasma membrane in the polar TE cells was lower than that
136 in the mural cells (Figure 1G left). In addition, the accumulation of F-actin at the cell
137 membrane was also more evident in mural cells, which expanded more than polar TE
138 cells (Figure 1G right). Since polar TE cells attach to the crowded ICM, these cells may
139 be exposed to lower tensions.

140 These results are consistent with our previous finding that ZO-1 condensates decrease
141 in the *Xenopus* ectodermal cells exposed to a higher tensile force (Hashimoto et al., 2019;
142 Kinoshita et al., 2020). Those findings suggest that the embryonic cells of E4.5 embryos,
143 especially TE cells on which more potent tensile forces than those of E3.5 embryos are
144 applied due to the inner pressure of the growing blastocyst cavity, might have reduced the

145 number and volume of ZO-1 puncta in the cytoplasm.

146

147 ***ZO-1 condensate is regulated by a force-dependent mechanism***

148 It is known that developing mouse embryos from E4.0 to E4.5 experience a gradually
149 increasing magnitude of luminal pressure due to expansion of the blastocyst cavity in the
150 presence of the ZP (Chan et al., 2019; Leonavicius et al., 2018). Accordingly, the cortical
151 tension of TE increases from E3.5 to E4.5 (Chan et al., 2019). To confirm that the
152 disappearance of ZO-1 condensates from the cytoplasm is dependent on a mechanical
153 force (inner hydraulic pressure of blastocyst cavity), using ouabain, we inhibited Na⁺/K⁺
154 ATPase which promotes the influx of water and therefore increases the volume of the
155 blastocyst cavity (Figure 2A, B). In these ouabain-treated embryos, significant numbers
156 of ZO-1 condensates were retained in the cytoplasm of the mural cells of E4.5 embryos
157 (Figures 2C-E), demonstrating that the cells released from the tension failed to trigger the
158 dissolution of the ZO-1 condensates.

159 Next, we mechanically reduced tension by releasing the blastocoel fluid of the ZO-1-
160 EGFP-expressing embryos by piercing embryos with a glass needle. Immediately after
161 they were pierced, embryos shrank and membrane localization of ZO-1 was reduced,
162 although it recovered within several hours (Figure 2F, G and Video S2). Immunostaining
163 with the anti-ZO-1 antibody confirmed that endogenous ZO-1 also behaves in a similar
164 way, observing more cytoplasmic puncta in pierced E4.5 embryos than in control E4.5
165 embryos (Figure S2). Together, these results suggest that ZO-1 condensate deforms in a
166 tensile force-dependent manner.

167

168 ***Cell type-dependent condensation of ZO-1***

169 To investigate the nature of the ZO-1 condensate, we performed a series of studies using
170 *Xenopus laevis* A6 cells in which full-length and mutant forms of GFP-ZO-1 were
171 transiently expressed for live-imaging (Figure 3A). In non-confluent A6 cells, full-length
172 GFP-ZO-1 localized mostly in the cell periphery and partially colocalized with F-actin
173 bundles (Figure 3B). It was intriguing, however, that in A6 cells, when GFP-ZO-1, which
174 lacks an actin-binding domain (Fanning et al., 2002) (GFP-ZO-1 Δ ABD), was expressed,
175 ZO-1 condensates became evident and formed large droplet-like structures with a smooth
176 surface (Figure 3C). This suggests that the formation of condensate is normally restricted
177 by the binding of ZO-1 to F-actin (Beutel et al., 2019; Schwyer et al., 2019) and that

178 GFP-ZO-1 Δ ABD lost its capacity for the interaction. In addition, we expressed the N-
179 terminal half and the C-terminal half of ZO-1 tagged with GFP (Figure 3A; GFP-ZO-1 Δ C
180 and Δ N, respectively). Of note, the N-terminal half contains two IDRs while the C-
181 terminal half has one relatively long stretch of IDR. We found that the N-terminal half is
182 sufficient to form condensates in the cytoplasm (Figure 3D).

183 Supporting this notion, co-expression with a red fluorescent protein fused to F-actin-
184 binding peptides, Lifeact, Utrophin and the actin-binding domain of Moesin
185 (MoesinABD) similarly induced condensates that were indistinguishable from those
186 induced by GFP-ZO-1 Δ ABD (Figure 3E). We also observed that the MoesinABD-
187 induced ZO-1 condensates dynamically moved, sometimes fused and underwent fission
188 (Video S3 and Figure S3), reminiscent of typical liquid droplets. Based on these results,
189 we speculate that the interaction of ZO-1 with F-actin might block the formation of large
190 condensates. We thus treated cells with Latrunculin B, a potent inhibitor of actin
191 polymerization, and found that the formation and extinction of cytoplasmic ZO-1
192 condensates depended on the development and destruction of the F-actin network (Figure
193 3F and Video S4). These observations clearly indicate that the efficiency and growth of
194 ZO-1 condensation are negatively regulated by the interaction with F-actin in A6 cells.

195 We next expressed GFP-ZO-1 in canine MDCK cells, which formed an epithelial-like
196 cell sheet when they reached confluence. In sharp contrast to A6 cells, naïve MDCK cells
197 in a non-confluent sparse culture displayed evident condensates in the cytoplasm (Figure
198 4A). The average size of the ZO-1 condensate in A6 cells co-expressing MoesinABD and
199 the naïve MDCK cells visualized by immunostaining were 0.8 μ m and 0.7 μ m,
200 respectively (Figure 4B). These observations indicate that the condensation and
201 localization of ZO-1 proteins depend largely on cell type. We then analyzed the properties
202 of ZO-1 condensates in these cells.

203

204 ***Dynamics of ZO-1 condensation in cultured cells***

205 To examine whether the ZO-1 condensates observed in the cultured cells are indeed the
206 products of LLPS, we first treated A6 cells harboring GFP-ZO-1 puncta by co-expressing
207 MoesinABD with digitonin, a cell-permeabilizing reagent that disrupts the equilibrium
208 between the condensate and the cytoplasm. That treatment shrank the condensates within
209 several minutes in A6 cells (Figure 4C and Video S5). GFP-ZO-1 puncta in MDCK cells
210 behaved in a similar way following digitonin treatment (Figure 4D and Video S6).

211 Furthermore, we performed FRAP analyses for the condensates in A6 cells expressing
212 MoesinABD or in naïve MDCK cells. In both A6 (+MoesinABD) and MDCK cells, after
213 photo-bleaching, fluorescence immediately started to recover, reaching approx. 50%
214 (Figure 4E and F). This quick and high recovery is a typical property of LLPS-mediated
215 condensates (Shiina, 2019). These results demonstrate that the ZO-1 puncta are droplet-
216 like condensates produced by LLPS and in equilibrium with the cytoplasm.

217

218 ***Disassembly of ZO-1 condensate is regulated by the establishment of robust cell-to-cell***
219 ***contact***

220 To test the possibility that the formation of ZO-1 condensate is influenced by the
221 mechanical environment in cultured cells as predicted from mouse embryogenesis, we
222 grew MDCK cells to form islands in a culture dish. In those islands, cells in the central
223 region reached confluence and formed a typical epithelial cell sheet. When we examined
224 the condensate formation in the confluent MDCK monolayer, it was notable that cells in
225 the central region lost the condensates, while the peripheral cells tended to have well-
226 grown condensates (Figure 5A and B). However, we occasionally observed cells that
227 harbor relatively large-sized condensates in the central region, but those cells were not
228 well attached to, and were free from, neighboring cells (data not shown). A similar
229 tendency was observed for endogenous ZO-1 protein detected by immunofluorescence
230 using the anti-ZO-1 antibody (Figure 5C). As shown in the graph, cells in the forefront
231 row (most outer layer) and the second row had 15 to 20 condensates per cell on average,
232 whereas cells in the inner or central region had only a few condensates (Figure 5D). One
233 feature that distinguishes the inner cells from peripheral cells is that after the cells reach
234 confluency, the former adopt polygonal shapes and have higher densities than the latter
235 (Figure 5C). This suggests that the inner cells establish a robust cell-to-cell contact by
236 tight and adherens junctions which is physically supported by the actin cytoskeleton and
237 an individual cell rigidly contacts with surrounding cells, resembling embryonic cells
238 under tensile force.

239 To support this idea, we conducted a laser ablation assay. MDCK cells were grown so
240 that the cells formed islands in one culture dish. Then the GFP-ZO-1 expression plasmid
241 was transfected and the plasma membrane of GFP-ZO-1-expressing cells was irradiated
242 with laser light. When cells inside of colonies that had ZO-1 at the cell junction were
243 irradiated, cells became stable after irradiation due to cell-cell contact with neighboring

244 cells (Figure 5E and Video S7). When peripherally-located cells which had ZO-1 puncta
245 were irradiated, those cells immediately erupted and disappeared after irradiation (Figure
246 5F and Video S8). These results suggest that in MDCK cells, the formation of ZO-1
247 condensate is negatively associated with the establishment of robust cell-to-cell contact.

248 To verify that idea, we reduced the cell-to-cell contact by treating MDCK cells
249 expressing GFP-ZO-1 with PBS containing trypsin-EDTA. When the trypsin-EDTA
250 solution was added, ZO-1 detached from the cell membrane and formed puncta as cells
251 lost their contact (Figure S4 and Video S9). This result further confirmed the idea that the
252 formation of ZO-1 condensate is regulated by cell-cell adhesion.

253 To further examine whether cell-to-cell contact negatively regulates ZO-1 condensate
254 formation in MDCK cells, we conducted a wound-healing assay using GFP-ZO-1–
255 expressing cells (Figure 6A-C and Video S10). Initially, most of the cells had few
256 condensates in a confluent cell sheet. After scratching, cells started to actively move
257 toward the wound site, forming a significant number of condensates. The FRAP assay
258 revealed that the fluorescent signals of GFP-ZO-1 condensates that formed during the
259 wound-healing assay recovered to about 40% within 10 min, suggesting that these
260 condensates were formed by LLPS (Figure S5A-C, Video S11 and S12). This result
261 indicates again that the formation of ZO-1 condensate is regulated by inter- and
262 extracellular environments such as cell-to-cell contact, which in turn suggests that the
263 cells that are harnessed with a well-developed F-actin network due to higher levels of
264 cell-to-cell adhesion may hamper ZO-1 condensation. In fact, cortical F-actin was well-
265 developed in the non-migrating polygonal cells far from the wound site, whereas
266 migrating cells around the wound site developed more stress fibers at the expense of
267 cortical actin (Figure 6D-F). This is consistent with our previous finding that *Xenopus*
268 embryonic cells lost ZO-1 condensates when cell-to-cell junctions were enhanced after
269 the application of force (Hashimoto et al., 2019; Kinoshita et al., 2020).

270 In addition, we found that hyper-osmolarity also induced the formation of ZO-1
271 condensate. When A6 cells expressing GFP-ZO-1 were treated with a high-salt medium,
272 ZO-1 formed puncta (Figure S5D and Video S13). They reversibly recovered when the
273 high-salt medium was washed out, indicating that ZO-1 LLPS is regulated by osmolarity.
274 As increased extracellular osmolarity led to a reduction in cell volume and loosening of
275 the cell membrane, this cellular phenotype may also be attributed to decreased membrane
276 tension and remodeling of F-actin.

277

278 **Discussion**

279 Our present study using mouse embryos as well as cultured cells, together with a
280 previous study with *Xenopus* embryos, indicate that similar mechanisms may govern
281 cellular responses to force across species. Briefly, mechanical stresses induced by forces
282 generated by dynamic morphogenesis and/or vigorous cell movements during
283 development, such as gastrulation, activate the force-dependent pathway leading to the
284 remodeling of cell-to-cell junctions. The present study also suggests that the process
285 involves the negative regulation of LLPS of ZO-1, leading to the collapse of ZO-1
286 condensates in the cytoplasm. Since embryonic tissues, as well as some adult tissues such
287 as heart and blood vessels, are constantly exposed to various forces including tensile force,
288 shearing force, etc., and since ZO-1 is a structurally conserved protein among species, we
289 propose that this mechanism may be employed to maintain the robustness and integrity
290 of tissues against various forces in general, and could also be an evolutionary innovation
291 when multicellular animal species such as placozoa emerged (Gonzalez-Mariscal et al.,
292 2011). Although our previous study using *Xenopus* embryos identified that the function
293 of the FGF receptor is essential for the force-induced pathway, it also suggested that FGF
294 ligands are not required for signal activation, shedding light onto the as yet unrevealed
295 activation mechanisms for FGFRs and other receptor tyrosine kinases (RTKs) by force.
296 In fact, epidermal growth factor receptor (EGFR), which is an upstream RTK for Erk,
297 was implicated in the mechanical force-dependent activation of Erk in the collective
298 migration of wound-healing cells (Hino et al., 2020). In both cases, cell deformation by
299 tensile forces appears to be the trigger of these pathways, supporting the idea that
300 mechanoresponsive signaling pathways may be highly conserved.

301 Interestingly, our present study using cultured cells demonstrated that the efficacy of
302 ZO-1 condensation as droplets largely depends on cell type, with MDCK cells forming
303 droplets in standard culture conditions whereas A6 cells only did so when the actin-
304 binding domain of ZO-1 was genetically deleted (Δ ABD), actin-polymerization was
305 inhibited, or actin was masked by its binding chemicals. These results suggest that the
306 disintegration of ZO-1 condensates in the cytoplasm is regulated by the interaction
307 between ZO-1 and F-actin, which in turn suggests that the difference between the amount
308 and dynamics of F-actin may distinguish these two cell types. Conversely, the revelation
309 of ZO-1 from F-actin is critical for maintaining large-sized ZO-1 condensate. These

310 notions further suggest that the control of ZO-1 re-localization from the cytoplasm to tight
311 junctions is dependent on the remodeling of cytoskeletal actin. At present, it is unclear
312 how the FGFR/Mek/Erk signal triggers or is related to the remodeling of actin and leads
313 to the accumulation of ZO-1 to the junction, but it is assumed that the signal is converted
314 into F-actin remodeling or ZO-1 phosphorylation, or both, as discussed next.

315 First, Erk might be able to regulate F-actin polymerization through small G proteins
316 such as GEF-H1 and its downstream target RhoA (Fujishiro et al., 2008; Itoh et al., 2014;
317 Ren et al., 1998). As described above, it was recently reported that Erk is activated in
318 collectively migrating MDCK cells by EGFR and regulates F-actin organization and
319 cellular contraction (Hino et al., 2020). Second, it is also possible that the cascade of
320 phosphorylation initiated by FGFR might eventually result in the phosphorylation of ZO-
321 1 protein, which has a number of potential phosphorylation sites for Ser/Thr kinases,
322 raising the possibility that phosphorylation of ZO-1 by Erk or other kinases may be a key
323 event for condensate dissolution. In fact, our previous report identified one particular site
324 in ZO-1 (Ser278) in the N-terminal half containing two putative IDRs that are sufficient
325 to form condensate, which was phosphorylated immediately after the application of force
326 and dephosphorylated within an hour (Hashimoto et al., 2019). Functional examination
327 of a possible link between RTK signaling and F-actin remodeling and a detailed
328 investigation of ZO-1 phosphorylation sites, which are sensitive to force and regulate
329 condensation, would provide useful information to clarify the force-dependent pathway.

330 What is the physiological significance of ZO-1 condensate formation? Why does ZO-1
331 need to be compartmentalized into the condensate? This may be too premature to discuss
332 before the nature of the ZO-1-containing condensate is fully understood, but we would
333 like to propose that the condensate serves as a reservoir for ZO-1 protein when cells are
334 relatively relaxed. Then, during development when the cells become stretched by tensile
335 force leading to the activation of Erk and the remodeling of F-actin, ZO-1 condensate
336 undergoes dissolution, and ZO-1 protein is delivered to the tight junction to exert its
337 function. It has been reported that tension mediates the remodeling of cell junctions (Ito
338 et al., 2017). Phase separation of ZO-1 may be directly involved in this regulatory system.
339 In this scenario, it is possible that LLPS acts as an on-demand ON/OFF switch of ZO-1
340 supply to the cell membrane from cytoplasm. This “reservoir mechanism” for ZO-1 may
341 be shared and employed by multicellular animal species and its evolutionary origin is an
342 intriguing problem worth studying in the future.

343 ZO-1, which is an essential component of the tight junction, contributes to establishing
344 cell-to-cell adhesion and is therefore implicated in various pathologies, including cancer.
345 Particularly, in EMT, the loss of cell-to-cell adhesion is known to drive the progress and
346 metastasis of cancer. It is also known that in neuronal cells, some condensates which
347 contain proteins such as the RNA-binding protein FUS (Murray et al., 2017) and tau
348 implicated in Alzheimer's disease (Wegmann et al., 2018) become aggregated depending
349 possibly on their concentrations, some time after their formation and/or their chemical
350 modifications. Those proteins are irreversibly aggregated and become dysfunctional,
351 attenuating the neuronal activity of the cells. Collectively considering these observations,
352 we speculate that impairment of the normal regulation of the LLPS of ZO-1 due to
353 changes in intracellular or extracellular conditions could be a possible cause for cancer
354 pathology. Therefore, in addition to embryogenesis, investigating the behavior of ZO-1
355 in adult tissues, especially in pathological conditions, would deepen the understanding of
356 the physiological significance of the ZO-1 reservoir in cells.

357

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374

375 **Figure legends**

376

377 **Figure 1. Change of ZO-1 localization in mouse hatching embryos.**

378 **A.** Schematic diagram of the hatching process of the mouse embryo. At E3.5, the
379 embryo is covered with the zona pellucida (ZP). During hatching, the embryo is
380 enlarged with an expansion of trophectoderm (TE) and emerges from the ZP. **B.**
381 Immunofluorescence of mouse E3.5 and E4.5 embryos. Embryos were stained with an
382 anti-ZO-1 antibody, Hoechst 33342 and Alexa Fluor 546 Phalloidin. Scale bar, 20 μ m.
383 The inset in the E3.5 phalloidin image was acquired with higher laser power,
384 demonstrating that the structure of cortical F-actin is formed at E3.5 even though the
385 signal intensity was weaker than that in the E4.5 embryo. The bottom panels are
386 enlarged images from the middle panels indicated by white squares. **C - G.** Quantitative
387 analyses of mouse embryos. The nuclear-dense inner cell mass (ICM) region was
388 determined by Hoechst staining. ‘Polar’ indicates TE to which ICM was attached, and
389 ‘mural’ includes TE to which ICM was not attached. 4 - 5 embryos were analyzed for
390 each condition. **C.** The signal intensities of ZO-1 at the cell periphery of the mural TE at
391 E3.5 and E4.5 were quantified. **D.** The number of particles in one TE cell was counted.
392 **E.** The areas of the TE cells were quantified. **F.** Fluorescence intensity of whole
393 embryos stained with the anti-ZO-1 antibody and phalloidin was measured. For
394 comparison, intensities were normalized by Hoechst staining intensity. $n = 5$ embryos.
395 **G.** The signal intensities of ZO-1 and phalloidin at the cell periphery of the mural and
396 polar TE of E4.5 embryos were quantified. n.s.: not significant. $*p < 0.01$.

397

398 **Figure 2. Expansion of TE regulates ZO-1 localization.**

399 **A and B.** Embryos were obtained at E3.5 and cultured for 24 h in the absence (A) or
400 presence of 0.3 mM ouabain (+Ob) (B). Embryos were immunostained with the anti-
401 ZO-1 antibody (left) and the images were analyzed using particle analysis in Image J
402 (right). **C-E.** The percentage of the particle area (C), number of particles (D) and cell
403 size (E) of E4.5 mural TE cells were quantified and plotted. **F.** E4.5 mouse embryos
404 expressing ZO-1-GFP were pierced with a glass needle. Pierced embryos shrank
405 immediately and the recovery process was imaged. **G.** The fluorescence intensity of
406 GFP-ZO-1 at the cell periphery at 0 h and 6 h were quantified. The X-axis indicates
407 distance from the cell membrane. Scale bars, 20 μ m. $*p < 0.01$.

408

409 **Figure 3. ZO-1 localization is regulated by F-actin in A6 cells.**

410 **A.** Domain structure of ZO-1 protein and expression constructs used here, GFP-tagged
411 full-length human ZO-1 (GFP-ZO-1), actin-binding domain (ABD)-deletion mutant
412 (GFP-ZO-1 Δ ABD), GFP-ZO-1 Δ C and GFP-ZO-1 Δ N. IDRs were predicted by PrDOS
413 (protein disorder prediction system) (Ishida and Kinoshita, 2007). **B.** GFP ZO-1 was
414 transiently expressed in *Xenopus laevis* A6 kidney cells. Cells were fixed and stained
415 with fluorescent phalloidin. **C.** Localization of GFP-ZO-1 and GFP-ZO-1 Δ ABD. **D.**
416 Localization of GFP-ZO-1 Δ C and Δ N. **E.** GFP-ZO-1 was co-expressed with actin-
417 binding constructs tagged with a red fluorescent protein. Lifeact-RFP, mCherry-
418 Utrophin and RFP-MoesinABD (actin-binding domain). **F.** A6 cells expressing GFP-
419 ZO-1 were treated with 0.5 μ M latrunculin B for 20 min, which was then washed out.
420 Scale bars, 20 μ m.

421

422 **Figure 4. Dynamics of GFP-ZO-1 particles expressed in A6 and MDCK cells.**

423 **A.** GFP-ZO-1 was transiently expressed in MDCK cells. When MDCK cells were
424 sparsely distributed, GFP-ZO-1 formed cytoplasmic puncta. When GFP-ZO-1 was
425 expressed in densely cultured MDCK cells, ZO-1 localization was different (see Figure
426 6A and the main text). In this figure, sparsely-distributed cells were shown. **B.** The size
427 of ZO-1 puncta in A6 cells co-expressed with MoesinABD and in MDCK cells were
428 compared. **C** and **E.** GFP-ZO-1 in A6 cells co-expressing MoesinABD. **D** and **F.** GFP-
429 ZO-1 in MDCK cells. **C** and **D.** Cells expressing GFP-ZO-1 were semi-permeabilized
430 with 0.012% digitonin. The size of puncta was quantified with Image J particle analysis.
431 $n = 12$ and 6 puncta, respectively. **E** and **F.** Fluorescent recovery after photobleaching
432 (FRAP) assay. The recovery of fluorescence was quantified. $n = 20$ puncta. Scale bars,
433 20 μ m.

434

435 **Figure 5. Dynamics of ZO-1 puncta in MDCK and A6 cells.**

436 **A** and **B.** GFP-ZO-1 was expressed in MDCK cells, forming islands. In cells located
437 inside of islands (indicated by arrowheads), GFP-ZO-1 was localized mainly at the
438 plasma membrane. In contrast, in cells at the periphery or outside of the colony
439 (indicated by arrows), GFP-ZO-1 formed puncta. **C.** MDCK cells that formed a colony
440 were immunostained with the anti-ZO-1 antibody. Images were analyzed using ‘particle

441 analysis' in Image J software. **D.** Cells in the island were divided into three groups with
442 respect to their location, namely the first and second rows from the edge, and the central
443 region of the island. The number of particles in each cell was counted. $n = 20$ cells. $*p <$
444 0.01 . **E-G.** Laser ablation of MDCK cells expressing GFP-ZO-1. **E.** Cells inside of an
445 island which had GFP-ZO-1 around the cell membrane. About 80% of these cells
446 remained stable after ablation (Video S7). **F.** A cell located close to the edge of an
447 island, and which had GFP-ZO-1 puncta. About 80% of these cells rapidly erupted and
448 detached from the bottom of the dish (Video S8). Arrowheads indicate laser ablation
449 sites. **G.** A statistical summary of the laser ablation experiment. "attached" indicates
450 cells that stayed attached to the dish after ablation, as shown in **E**. "erupted" indicates
451 cells that erupted after ablation and detached from the dish, as shown in **F**. Data were
452 obtained from two experiments. The total numbers of cells for the laser ablation were 26
453 inside and 24 outside cells. Scale bars, 20 μm .

454

455 **Figure 6. Wound-healing assay using MDCK cells expressing GFP-ZO-1**

456 **A and B.** The confluent cell sheet was scratched and observed. Just after scratching (0
457 h), GFP-ZO-1 was at the cell periphery, whereas after 3 hours (3 h), it formed puncta in
458 many of the cells close to the scratch (within 200 μm). Cells in **B** were magnified from
459 #1 and #2 cells in **A**. Scale bar, 20 μm . **C.** The number of cells with GFP-ZO-1 particles
460 were counted. Cells located within 200 μm from the scratch site were defined as "edge"
461 cells. Cells more than 200 μm away from the scratch site were defined as "inner" cells.
462 The data were obtained from three experiments. **D and E.** Inner and edge cells were
463 fixed 10 h after scratching and stained with phalloidin. The arrow indicates the wound
464 site. Scale bar, 20 μm . **F.** Fluorescence intensity of phalloidin staining at the plasma
465 membrane was measured. $*p < 0.01$.

466

467 **Supplementary Figure and Video Legends**

468 **Figure S1. E3.5 and E4.5 embryos expressing ZO-1-EGFP (related to Figure 1)**

469 **A.** Embryos from mice expressing ZO-1-EGFP by EGFP-knock-in were observed.
470 Before hatching (E3.5), ZO-1 puncta were observed around the cell periphery, but cell-
471 cell boundaries were not clear. After hatching (E4.5), membrane localization of ZO-1-
472 GFP became evident. The signal around the nuclei in the TE and large puncta in the
473 ICM are autofluorescence, which was confirmed by observation of wild-type mouse
474 embryos not expressing ZO-1-GFP (data not shown). **B.** Mouse E3.5 and E4.5 embryos
475 expressing ZO-1-EGFP at higher magnification. **C.** Fluorescence intensity around cell
476 boundaries in ZO-1-EGFP-expressing embryos was quantified. After hatching, ZO-1-
477 EGFP localized to the plasma membrane and formed sharp boundaries with ZO-1-
478 EGFP. Scale bars, 20 μm .

479

480 **Figure S2. Immunofluorescence of mouse E4.5 embryos pierced with a glass needle**
481 **(related to Figure 2)**

482 **A.** Mouse E4.5 embryos expressing ZO-1-GFP were pierced with a glass needle, then
483 the volume of the blastocyst cavity was reduced, and embryos shrank. Those embryos
484 were fixed and immunostained. **B.** The number of particles was counted using Image J
485 particle analysis. **C.** The area of particles and cytoplasm of one mural TE cell was
486 measured by Image J software, and the ratio of those values was plotted. * $p < 0.01$.

487

488 **Figure S3. Dynamics of GFP-ZO-1 puncta in A6 cell co-expressing RFP-**
489 **MoesinABD (related to Figure 3)**

490 Puncta often underwent fission and fusion, as indicated by arrowheads. These images
491 are from Video S3.

492

493 **Figure S4. Trypsin-EDTA treatment of confluent growing MDCK cells**
494 **expressing GFP-ZO-1 (related to Figure 5)**

495 As cells lost their cell-cell contact and their shape became spherical, ZO-1 detached
496 from the plasma membrane and formed puncta. These images are from Video S9.

497

498 **Figure S5. Localization of GFP-ZO-1 in a wound-healing assay and in osmolarity**
499 **change in MDCK cells (related to Figure 6)**

500 **A-C. Wound-healing assay followed by FRAP assay.** **A.** Cells that formed GFP-ZO-1
501 puncta in the wound-healing assay. The cell indicated by the square and the arrowhead
502 was used for the following FRAP assay. **B** and **C.** GFP-ZO-1 puncta formed during
503 wound healing were subjected for the FRAP assay. Three cells were analyzed. **D.** An A6
504 cell expressing GFP-ZO-1 was treated with a high-salt medium (four-fold higher
505 concentration of PBS) for 40 min then returned to standard medium. Scale bars, 20 μ m.

506

507 **Video S1.** Mouse embryos expressing ZO-1-EGFP were flushed from uteri at one-day
508 post-coitum and observed by time-lapse microscopy for 92 h (related to Figure S1).

509

510 **Video S2.** Mouse E4.5 embryos expressing ZO-1-EGFP were pierced by a glass needle
511 and observed by time-lapse microscopy (related to Figure 2F).

512

513 **Video S3.** An A6 cell co-expressing GFP-ZO-1 and RFP-MoesinABD was imaged by
514 time-lapse microscopy for 885 min (related to Figure S3).

515

516 **Video S4.** Latrunculin B treatment of an A6 cell co-expressing GFP-ZO-1 and RFP-
517 MoesinABD (related to Figure 3F).

518

519 **Video S5.** A6 cells co-expressing GFP-ZO-1 and RFP-MoesinABD were treated with
520 digitonin ("digitonin" at 50 sec in the video) and imaged by time-lapse microscopy
521 (related to Figure 4C).

522

523 **Video S6.** An MDCK cell expressing GFP-ZO-1 was treated with digitonin
524 ("digitonin" at 50 sec in the video) and imaged by time-lapse microscopy (related to
525 Figure 4D).

526

527 **Video S7.** Laser ablation of MDCK cells expressing GFP-ZO-1 inside of the island. The
528 laser was irradiated at 4 sec at the membrane indicated by arrowheads (related to Figure
529 5E).

530

531 **Video S8.** Laser ablation of MDCK cells expressing GFP-ZO-1 at the periphery of the
532 island. The laser was irradiated at 4 sec at the membrane indicated by the arrowhead.

533 The cell erupted and disappeared immediately after irradiation (related to Figure 5F).

534

535 **Video S9.** MDCK cells expressing GFP-ZO-1 were treated with a trypsin-EDTA

536 solution. The video starts immediately after treatment (related to Figure S4).

537

538 **Video S10.** A wound-healing assay of MDCK cells. A sheet of MDCK cells transfected

539 with the GFP-ZO-1 construct was scratched and observed by time-lapse microscopy

540 (related to Figure 6).

541

542 **Video S11.** A wound-healing assay of MDCK cells, followed by a FRAP assay (1). A

543 sheet of MDCK cells transfected with the GFP-ZO-1 construct was scratched and

544 observed by time-lapse microscopy (related to Figure S5A). The cell for the following

545 FRAP assay was indicated by an arrowhead at the first and the last frames.

546

547 **Video S12.** A wound-healing assay of MDCK cells, followed by a FRAP assay (2).

548 FRAP assay was conducted using a cell in Video S11 which formed ZO-1 puncta

549 (related to Figure S5B). The granule bleached in the FRAP assay was indicated by an

550 arrowhead.

551

552 **Video S13.** A6 cell expressing GFP-ZO-1 was treated with a high-salt medium (four-

553 fold higher concentration of PBS) at the 4-min time point indicated by "+osmo" in the

554 video and then returned to normal medium at the 55-min time point indicated by "wash

555 out" (related to Figure S5D)..

556

557

Methods

558

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-ZO-1 antibody	Thermo Fisher Scientific	cat. # 33-9100; RRID:AB_253314 7
goat anti-mouse IgG antibody, Alexa Fluor 488	Thermo Fisher Scientific	cat. # A28175; RRID:AB_253616 1
Chemicals, Peptides, and Recombinant Proteins		
Blocking One	Nacalai Tesque	cat.# 03953
KSOM	Millipore	cat. # MR-106D
ouabain	Sigma	cat.# O3125
Alexa Fluor 546 Phalloidin	Thermo Fisher Scientific	cat.# A22283; RRID:AB_263295 3
Hoechst 33342	Thermo Fisher Scientific	cat.# H3570
Lipofectamine 2000	Invitrogen	cat. # 11668-027
Effectene	Qiagen	cat. # 301425
Latrunculin B	Enzo Life Science	cat. # BML-T110-0001
digitonin	WAKO	cat. # 048-02124
trypsin	Nacalai Tesque	cat # 35555-54
Experimental Models: Organisms/Strains		
<i>Xenopus laevis</i> (J-strain)	N/A	N/A
R26-ZO-1EGFP	RIKEN	accession # CDB026K
wild type ICR mice	Japan CREA	N/A
Experimental Models: Cell Lines		

MDCK I cells	Fujiwara et al. (2016)	N/A
A6 cells	Mimori-Kiyosue et al. (2007)	N/A
Recombinant DNA		
human ZO-1	Konno et al. (2008)	N/A
GFP-ZO-1/pCS2	This paper	N/A
GFP-ZO-1 Δ ABD/pCS2 (Δ 1151-1371)	This paper	N/A
GFP-ZO-1 Δ C/pCS2 (Δ 595-1748)	This paper	N/A
GFP-ZO-1 Δ N/pCS2 (Δ 1-594)	This paper	N/A
Lifeact-RFP	lioka et al. (2007)	N/A
RFP-MoesinABD	lioka et al. (2004)	N/A
mCherry-Utrophin	Kim and Davidson (2013)	N/A
Software and Algorithms		
Fiji	NIH	https://fiji.sc
Python 3.6	Python Core Team	https://www.python.org
Pandas	v0.22	http://pandas.pydata.org
seaborn	v0.8	https://seaborn.pydata.org
Matplotlib	v2.2	https://matplotlib.org
Andor IQ2	Oxford Instruments	https://andor.oxinst.com/products/iq-live-cell-imaging-software/
PrDOS (protein disorder prediction system)	(Ishida and Kinoshita, 2007)	http://prdos.hgc.jp/cgi-bin/top.cgi

560

561 **Lead Contact and Materials Availability**

562

563 Further information and requests for resources and reagents should be directed to and
564 will be fulfilled by the Lead Contact, Naoto Ueno (nueno@nibb.ac.jp.). All
565 unique/stable reagents generated in this study are available from the Lead Contact with
566 a completed Material Transfer Agreement.

567

568 **Method details**

569

570 **Mouse embryo collection**

571 Animal care and experiments were conducted in accordance with the Guidelines of
572 Animal Experimentation of the National Institutes for Natural Sciences. All animal
573 experiments were approved by the Animal Research Committee of the National
574 Institutes for Natural Sciences. Mice were maintained in a light- and temperature-
575 controlled room using a 12 h light:12 h dark cycle at 23 \pm 2 °C.

576 Males of R26-ZO-1EGFP (Katsunuma et al., 2016) or wild type ICR (Japan CREA)
577 were mated with wild type ICR females to obtain blastocysts. Preimplantation E3.5
578 embryos were flushed from uteri with KSOM (MR-106D, Millipore). Embryos were
579 cultured in KSOM covered with mineral oil at 37 °C and 5% CO₂. Fluorescent signals
580 were monitored using a confocal microscope (A1, Nikon) or a spinning disc confocal
581 microscope (CV1000, Yokogawa). For immunofluorescence, embryos were fixed at
582 4 °C in 4% paraformaldehyde (PFA) in PBS overnight. For ouabain treatment, 10 mM
583 ouabain (cat. # O3125, Sigma) in DMSO were diluted to the indicated concentrations.

584

585 **Manipulation of mouse embryos**

586 To remove the zona pellucida, embryos were treated with Tyrode's solution (Bradley,
587 1987). Embryos were transferred to a drop of Tyrode's solution. The zona was removed
588 typically within 1 min. Then, embryos were washed three times in KSOM. To pierce the
589 mural trophectoderm, microinjection needles were made from a 1 mm diameter glass
590 capillary (GD1, Narishige), the needle was set to a micromanipulator (Leica), and
591 embryos were pierced with the needle by manual manipulation.

592

593 **Immunofluorescence with mouse embryos**

594 Fixed embryos were washed three times with 1% bovine serum albumin (BSA) in PBS,
595 and then soaked in a blocking solution, 0.1% Triton X-100 in Blocking One (cat. #
596 03953, Nacalai, Japan) for 1 h at room temperature. Then, the embryos were incubated
597 in the primary antibody solution containing an anti-ZO-1 antibody (cat. # 33-9100,
598 Thermo) diluted 100-times in Blocking One at 4 °C overnight. After washing three
599 times with the blocking solution, embryos were incubated in a secondary antibody
600 solution containing 100-times diluted goat anti-mouse IgG antibody Alexa Fluor 488
601 (cat. # A27185, Thermo), 100-times diluted Alexa Fluor 546 Phalloidin (cat. # A22283,
602 Thermo Fisher), and 10 µg/ml Hoechst 33342 (cat. # H3570, Thermo Fisher) in
603 Blocking One. Fluorescent signals were monitored using Nikon A1 or Leica SP8
604 confocal microscopes.

605

606 **Measurement of fluorescent intensities and particle analyses**

607 Fiji/Image J was used to measure fluorescent intensities. Particle analyses in Fiji/Image
608 J were used to count the numbers of particles and measure particle areas. Images were
609 converted to an 8-bit type, and cytoplasmic regions were manually determined. The
610 "analysis particles" program was run with the threshold signal value set at 20, and
611 circularity was 0.25-1.0 for the definition of particles.

612

613 **GFP-ZO-1 expression constructs**

614 Human ZO-1 cDNA was a gift from Dr. Fumio Matsuzaki (Konno et al., 2008). EGFP-
615 human ZO-1/pCS2 was constructed by Dr. Makoto Suzuki. ZO-1ΔABD lacks amino
616 acid (aa) # 1151-1371. ZO-1ΔC lacks aa # 595-1748. ZO-1-1ΔN lacks aa # 1-594.
617 Lifeact-RFP is described in Iioka et al. (2007). MoesinABD is described in Iioka et al.
618 (2004). The mCherry-Utrophin construct was a gift from Lance A. Davidson (Kim and
619 Davidson, 2013).

620

621 **Cell culture and transfection of plasmids**

622 A6 cells, established from a normal *X. laevis* kidney, were a gift from Dr. Yuko Mimori-
623 Kiyosue (Mimori-Kiyosue et al., 2007). A6 cells were grown at 24 °C without CO₂ in
624 Leibovitz's L-15 medium (50% L-15 medium, 10% fetal bovine serum (FBS), 200 mg/l
625 kanamycin). MDCK cells were a gift from Dr. Mitsuru Nishita and Dr. Kensaku

626 Mizuno. MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM)
627 containing 10% FBS. Transfection was conducted using Lipofectamine 2000
628 (Invitrogen # 11668-027) for MDCK cells and Effectene (Qiagen # 301425) for A6 cells
629 following the manufacturers' instructions.

630

631 **Immunofluorescence of the tissue culture cells**

632 Cells were fixed in 4% PFA in PBS at 4 °C overnight, washed with 0.1% Triton X-100
633 in PBS. Blocking One (cat. # 03953, Nacalai Tesque, Japan) was used to block for 1 h at
634 room temperature, followed by incubation in the primary antibody solution, a 100-times
635 diluted anti-ZO-1 antibody (cat. # 33-9100, Thermo) in Blocking One at 4 °C overnight.
636 After washing three times in Blocking One, cells were incubated in the secondary
637 antibody solution containing 200-times diluted goat anti-mouse IgG antibody Alexa
638 Flour 488 (cat. # A27185, Thermo) and 200-times diluted Alexa Fluor 546 Phalloidin
639 (cat. # A22283, Thermo Fisher) in Blocking One at 4 °C overnight. After washing three
640 times in PBS, cells were observed using a Leica SP8 confocal microscope.

641

642 **Laser ablation**

643 Laser ablation was conducted using an Olympus IX 81 inverted microscope (20 x /0.70
644 NA dry objective lens), equipped with a spinning-disc confocal unit Yokogawa CSUX-1
645 and iXon3 897 EM-CCD camera (Andor), controlled with Andor IQ2 software. An N2
646 Micropoint laser (16 Hz, 365 nm, 2.0 μW, Photonic Instruments) was focused on the
647 membrane at the cell membrane. Time-lapse images were acquired every 200 msec
648 before and during the laser ablation process and analyzed with Fiji software.

649

650 **Fluorescence recovery after photobleaching (FRAP)**

651 FRAP assays in the tissue culture cells were conducted with a Leica SP8 confocal
652 microscope and software equipped in the microscope operating system. Regions of
653 interest (ROI) were bleached using a 488 nm laser. Pre-bleach and post-bleach images
654 were acquired with a 488 nm laser. Fluorescence recovery of GFP-ZO-1 was monitored.
655 Recovery data were background corrected and normalized to the ROI intensity before
656 bleaching. A reference ROI outside the bleached area was processed in the same way.

657

658 **Observation of GFP-ZO-1 dynamics in the tissue culture cells**

659 For the wound-healing assay, MDCK cells almost confluent grown on a glass-bottom
660 dish were transfected with GFP-ZO-1/pCS2. After one day, the cell layer was scratched
661 with a Pipetman tip, incubated at 37 °C, 5% CO₂ in a stage-top incubator (cat. # STXG,
662 Tokai HIT, Japan) and observed by a Leica SP8 confocal microscope.

663 For the latrunculin B treatment, 2.5 μM latrunculin B in the culture medium was added
664 to the culture dish of A6 cells transiently expressing GFP-ZO-1 so that the final
665 concentration of latrunculin B was 0.5 μM. For digitonin treatment, 0.06% digitonin in
666 the culture medium was added to the culture dish so that the final concentration of
667 digitonin was 0.012%. For hyperosmolarity treatment of A6 cells, 10 x PBS was added
668 so that the concentration of PBS changed from 0.5 x to 1.5 x. Cells were observed at
669 room temperature. For the trypsin-EDTA treatment, the culture medium of MDCK cells
670 was replaced with PBS containing 0.05% trypsin and 1 mM EDTA.
671

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673

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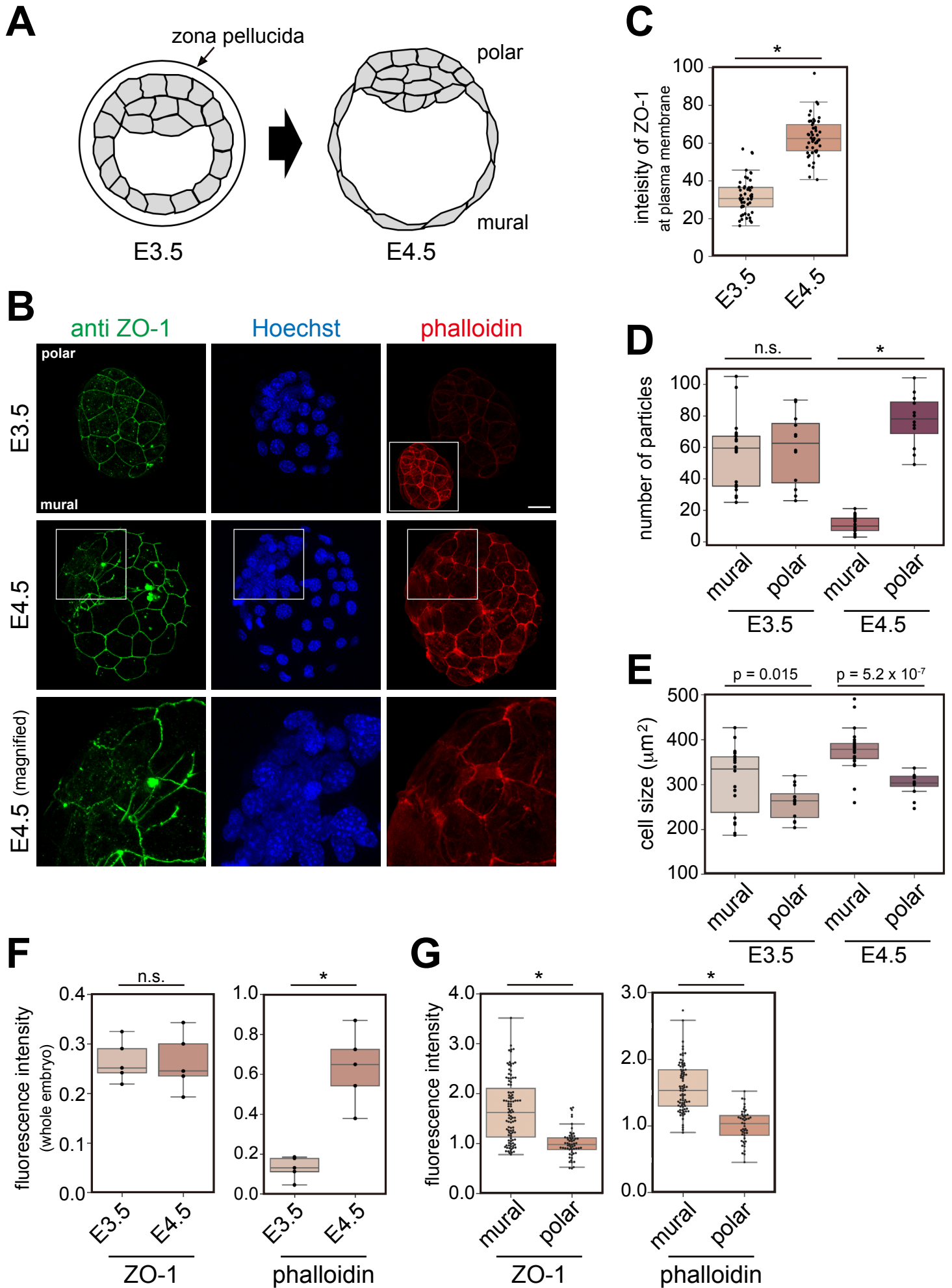
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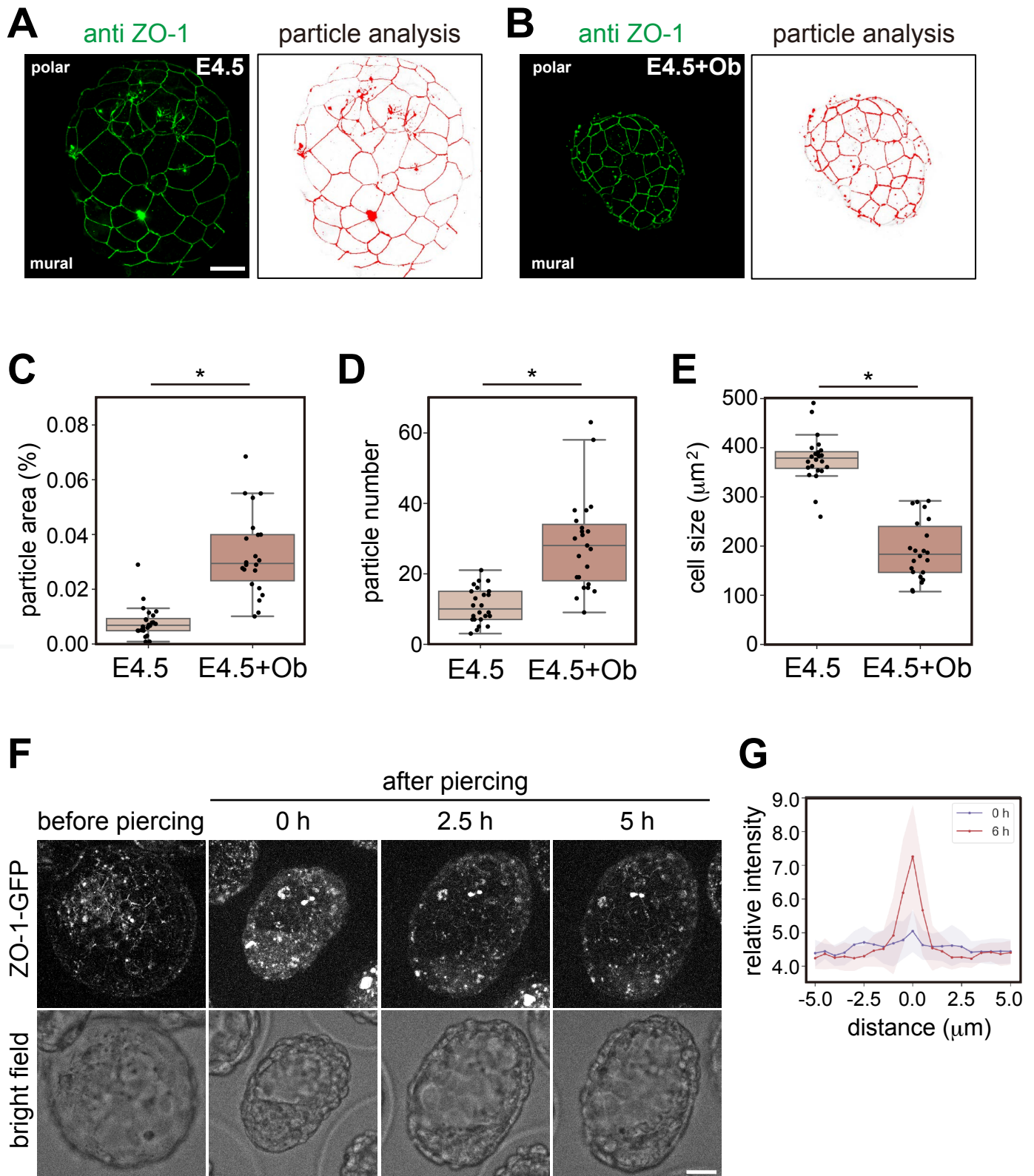
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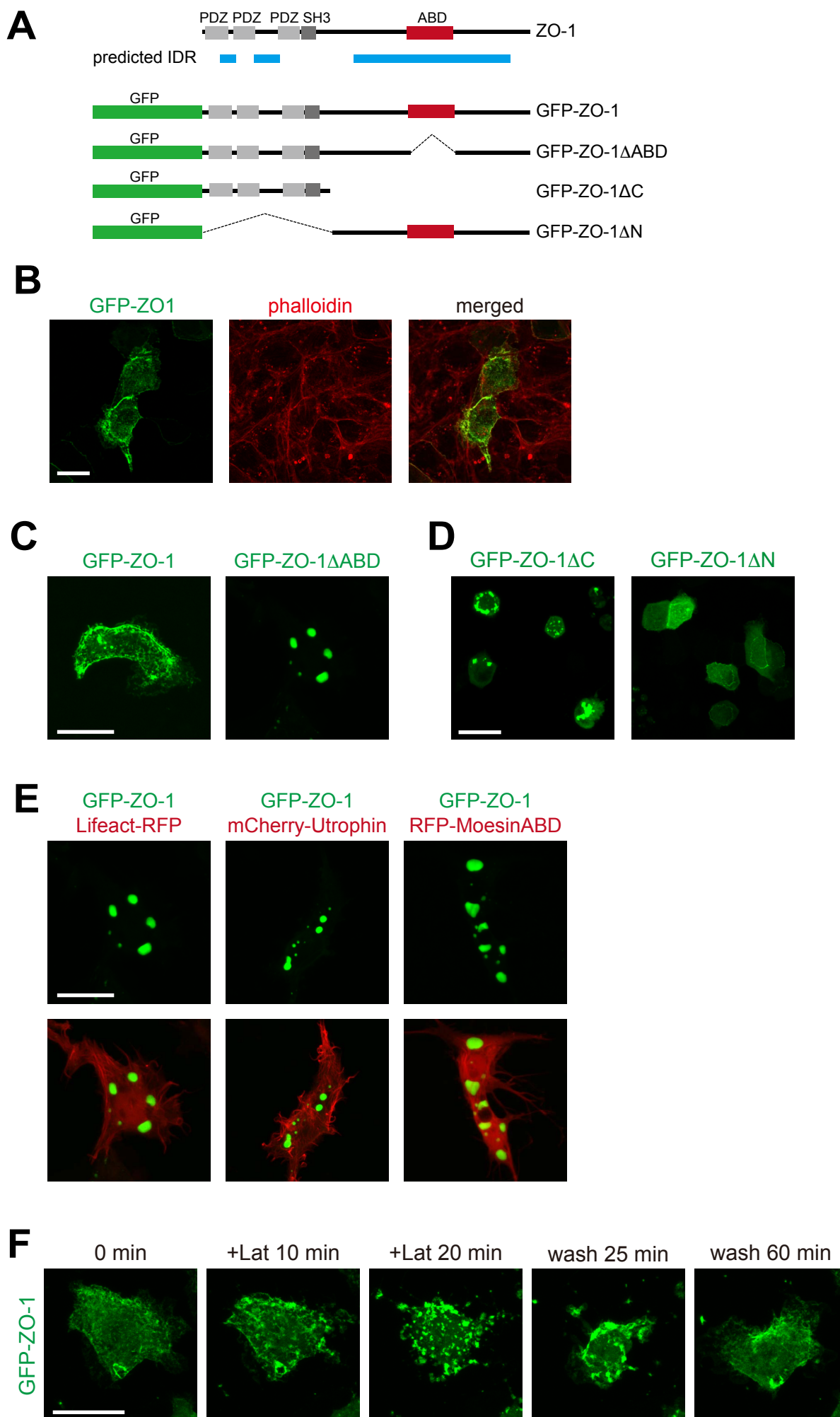
Kinoshita et al. Figure 1



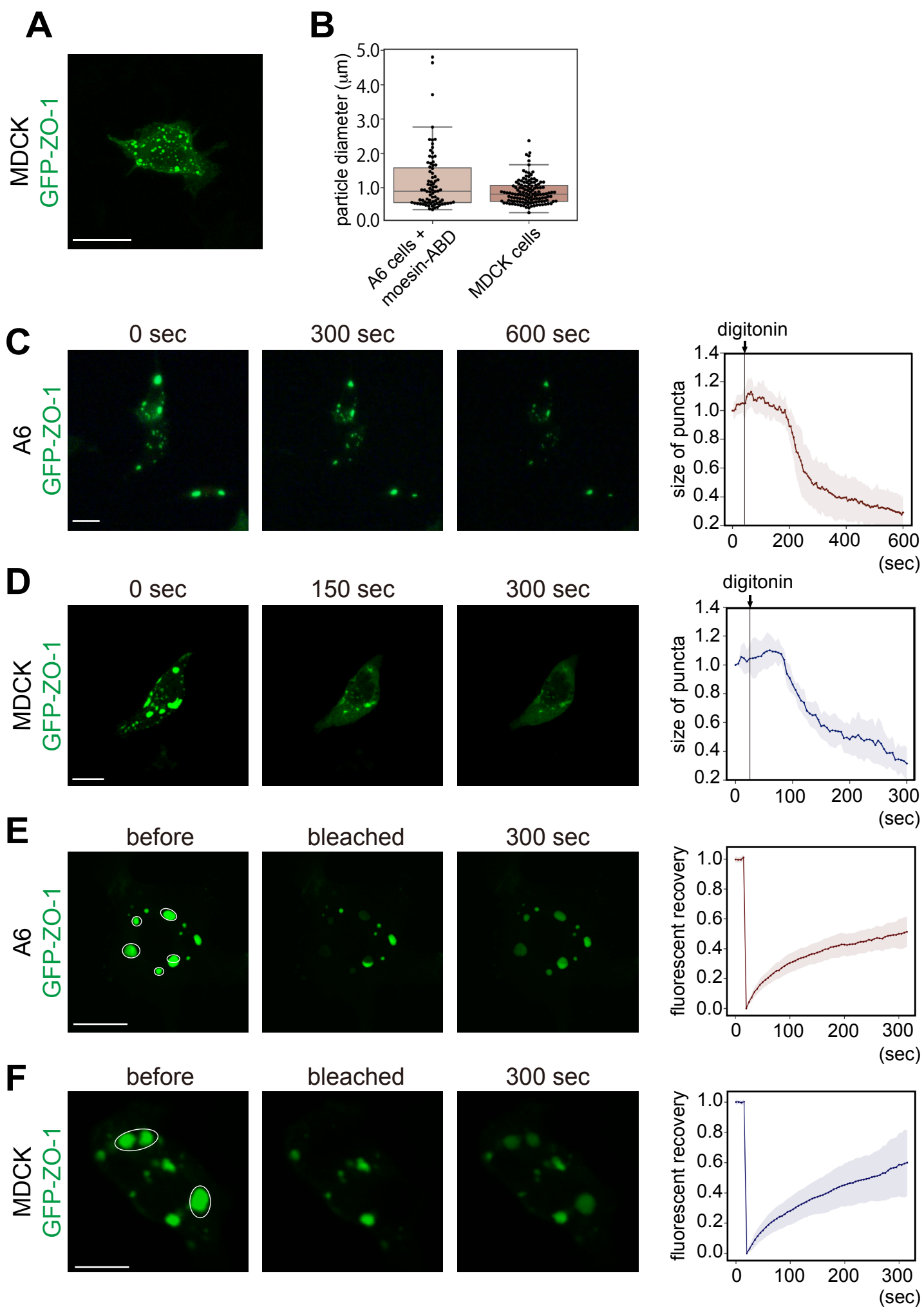
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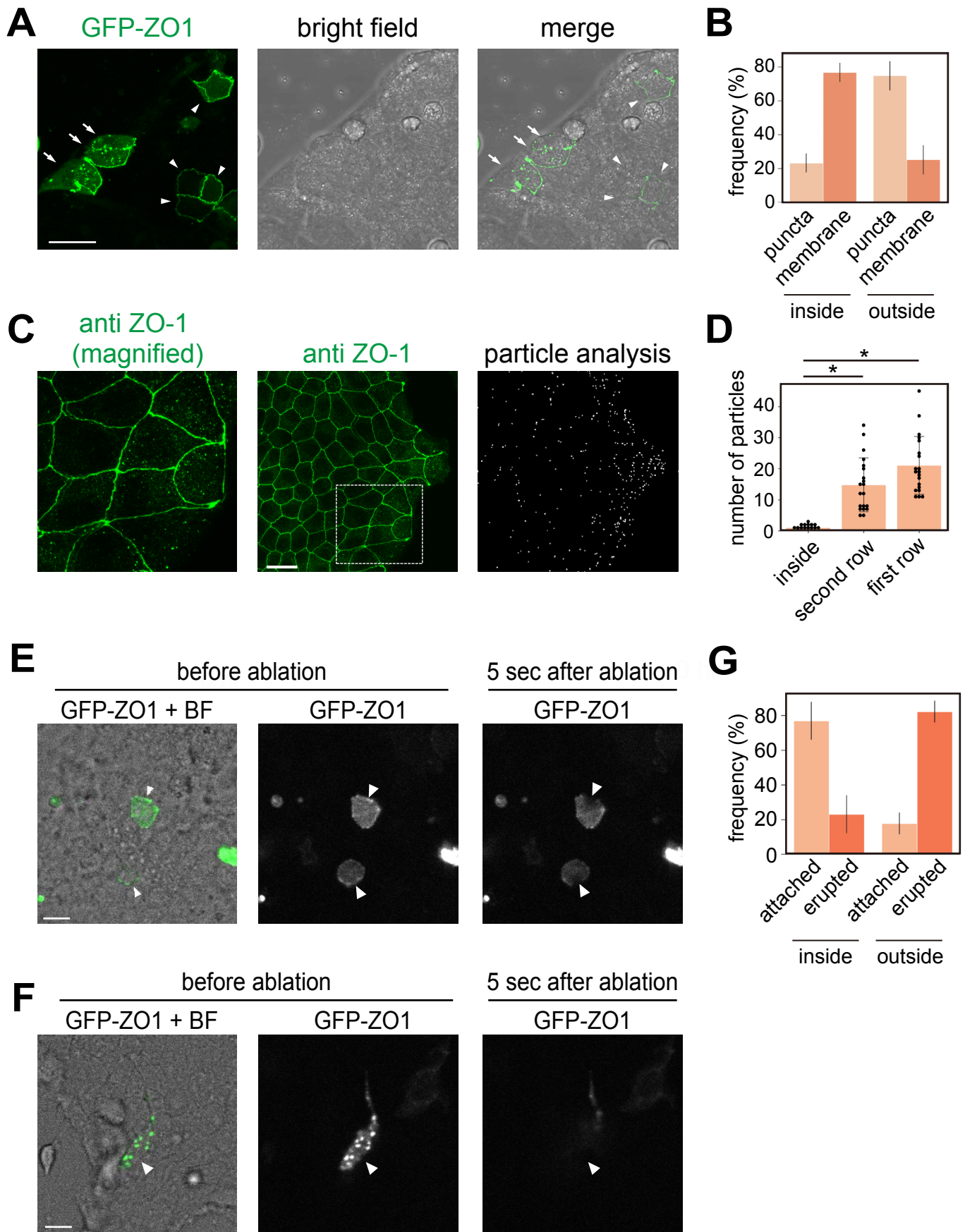
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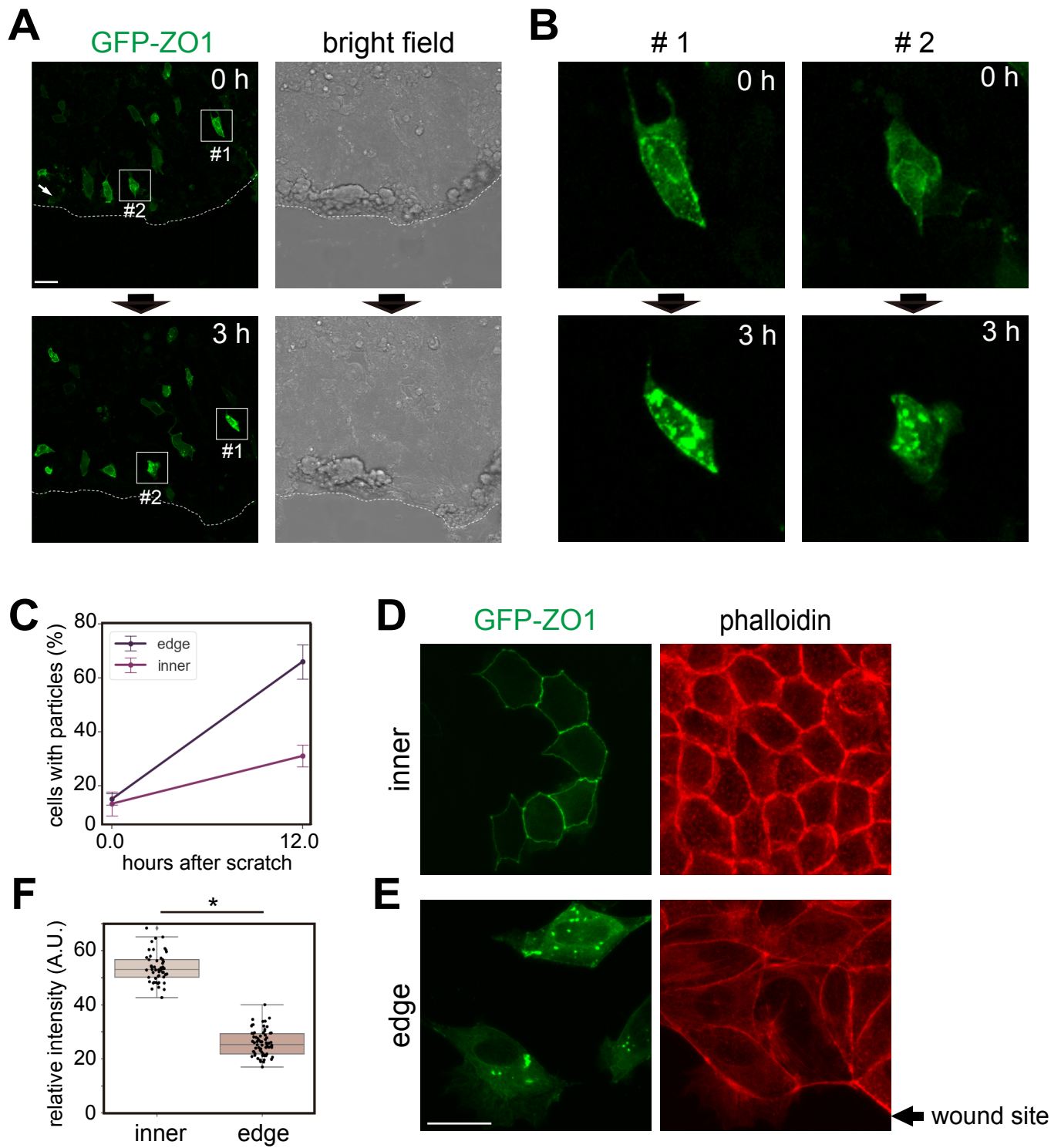
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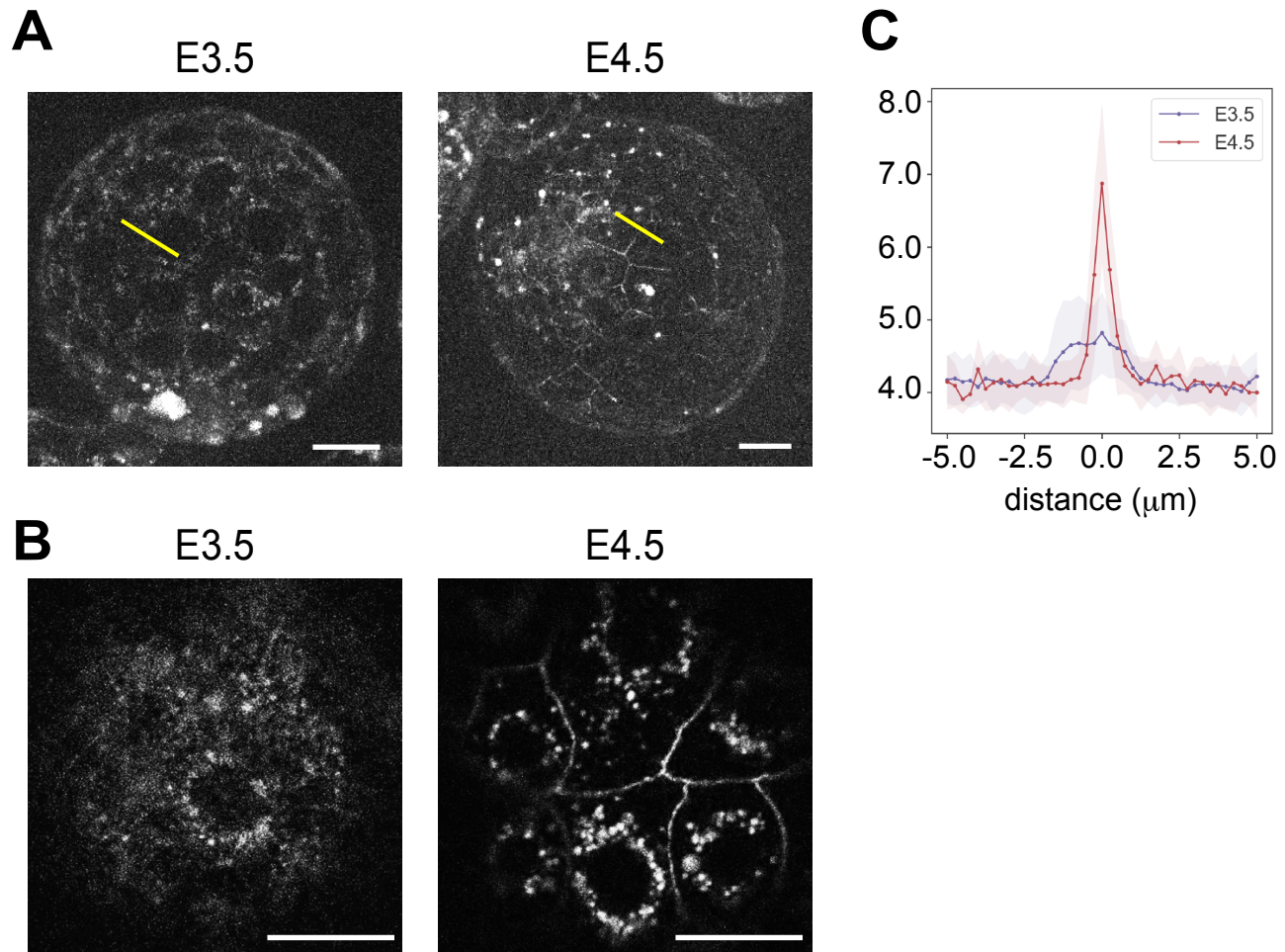
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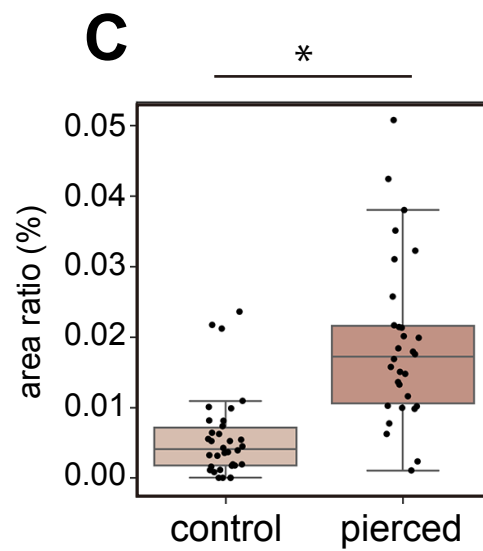
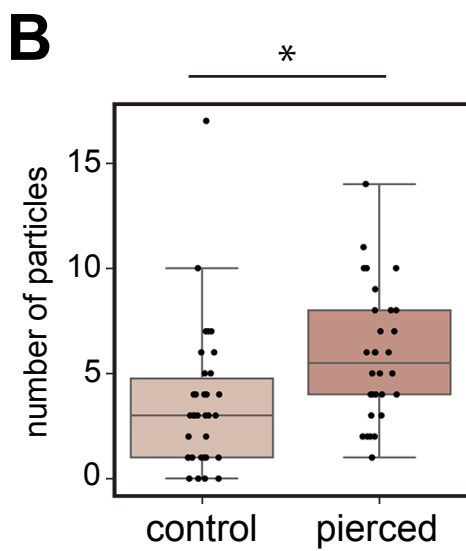
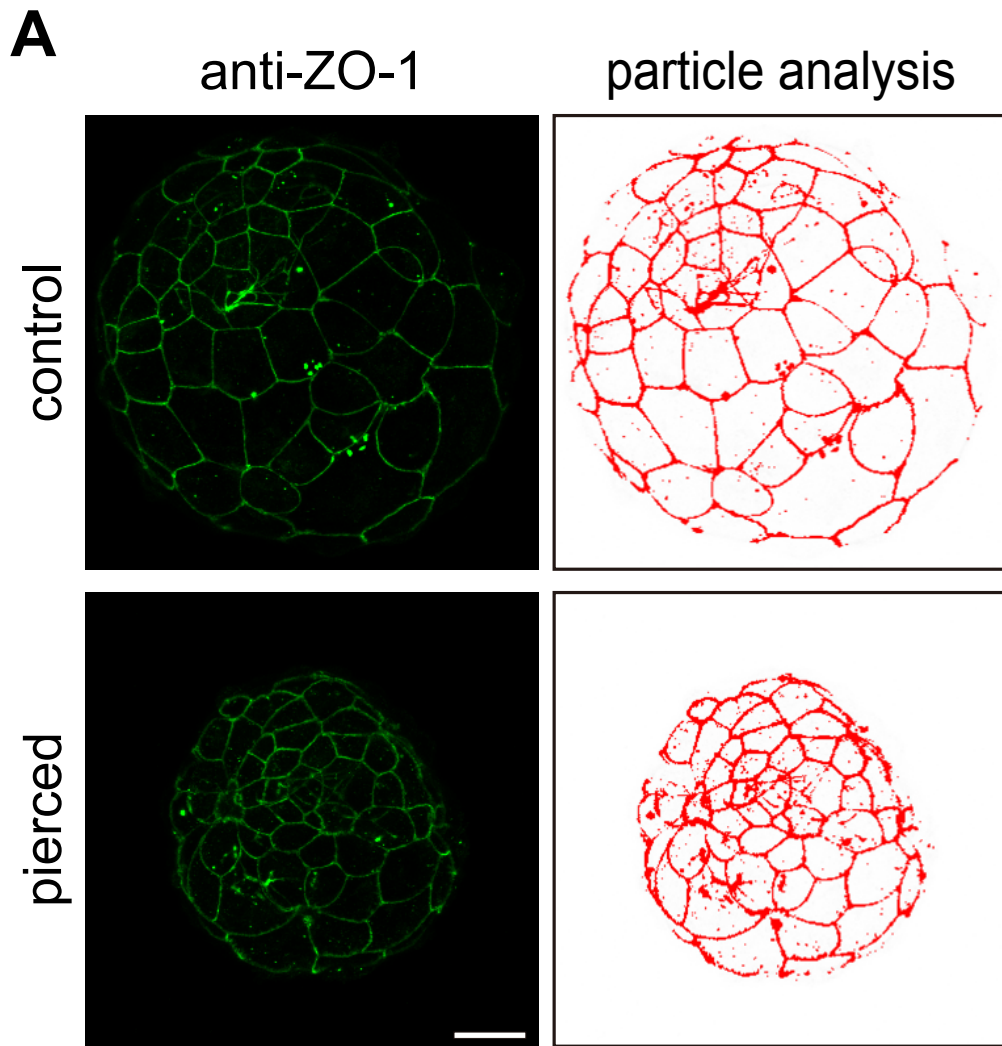
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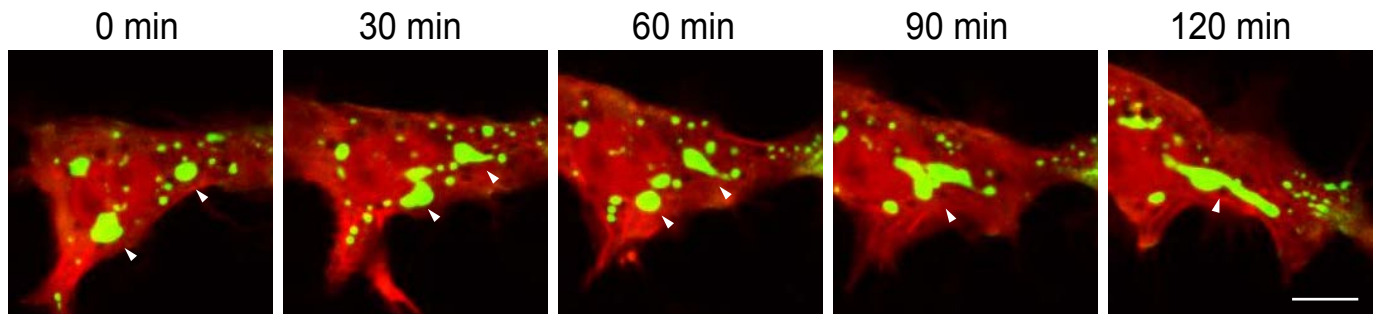
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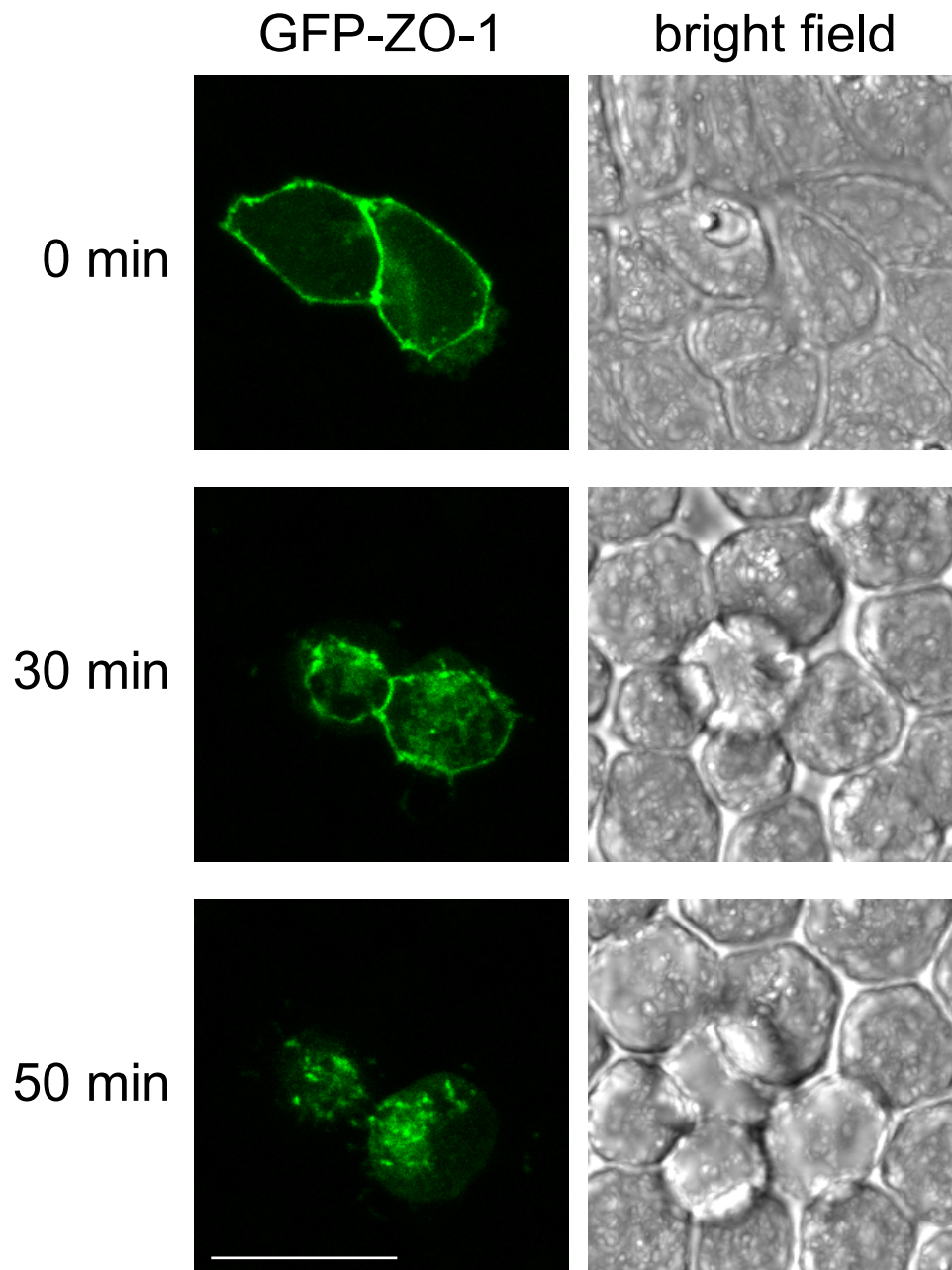
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Kinoshita et al. Figure S3

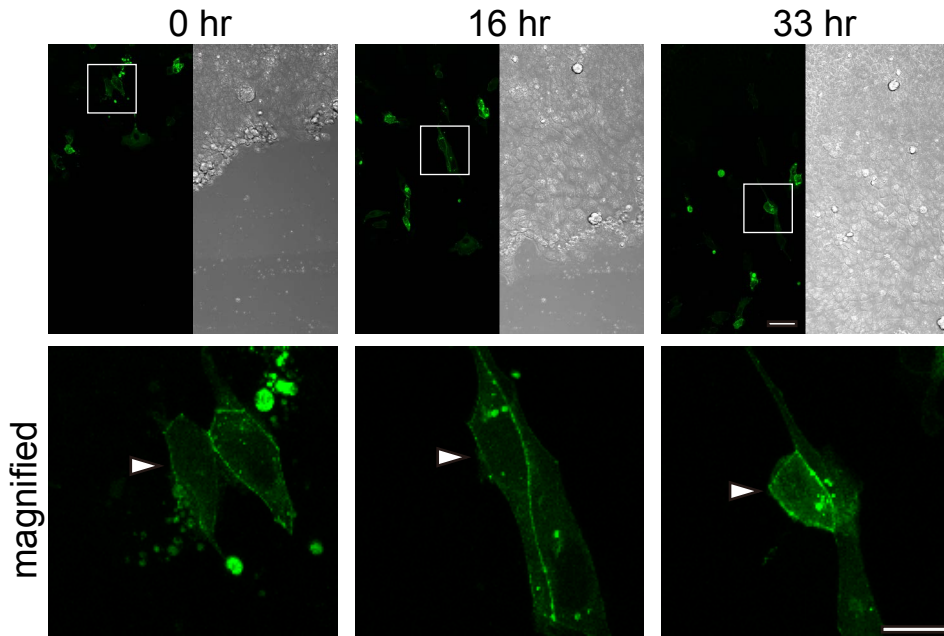


trypsin-EDTA treatment

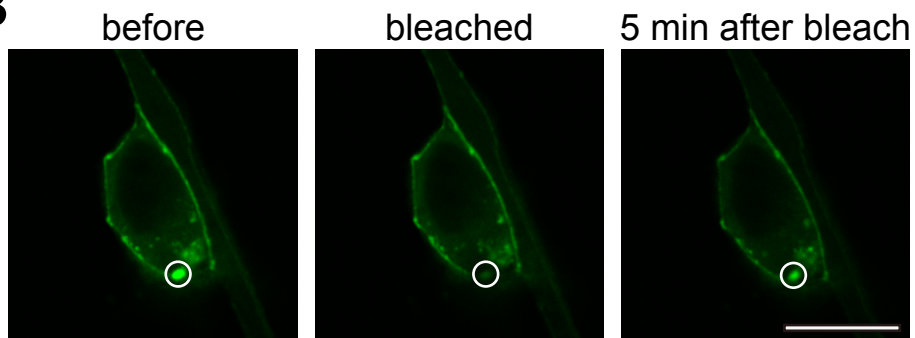


Kinoshita et al. Figure S5

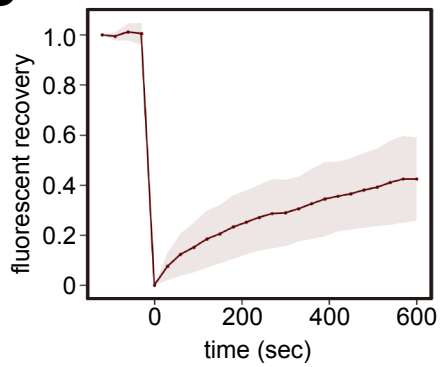
A



B



C



D

