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The nanoscale organization of the Wnt signaling integrator Dishevelled in the development-essential vegetal cortex domain of an egg and early embryo

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35 **Abstract**

36 Wnt/ β -catenin (cWnt) signaling is a crucial regulator of development and Dishevelled (Dsh/Dvl)
37 functions as an integral part of this pathway by linking Wnt binding to the frizzled:LRP5/6
38 receptor complex with β -catenin-stimulated gene expression. In many cell types Dsh has been
39 localized to ill-defined cytoplasmic puncta, however in sea urchin eggs and embryos confocal
40 fluorescence microscopy has shown that Dsh is localized to puncta present in a novel and
41 development-essential vegetal cortex domain (VCD). In the present study, we used super-
42 resolution light microscopy and platinum replica TEM to provide the first views of the
43 ultrastructural organization of Dsh within the sea urchin VCD. 3D-SIM imaging of isolated egg
44 cortices demonstrated the concentration gradient-like distribution of Dsh in the VCD, whereas
45 higher resolution STED imaging revealed that some individual Dsh puncta consisted of more
46 than one fluorescent source. Platinum replica immuno-TEM localization showed that Dsh
47 puncta on the cytoplasmic face of the plasma membrane consisted of aggregates of pedestal-
48 like structures each individually labeled with the C-terminus specific Dsh antibody. These
49 aggregates were resistant to detergent extraction and treatment with drugs that disrupt actin
50 filaments or inhibit myosin II contraction, and coexisted with the first division actomyosin
51 contractile ring. These results confirm and extend previous studies and reveal, for the first time
52 in any cell type, the nanoscale organization of plasma membrane tethered Dsh. Our current
53 working hypothesis is that these Dsh pedestals represent a prepositioned scaffold organization
54 that is important for canonical Wnt pathway activation at the sea urchin vegetal organization and
55 may also be relevant to the submembranous Dsh puncta present in other eggs and embryos.

56

57

58 **Introduction**

59 Wnt signaling controls a broad range of fundamental processes in cell and developmental
60 biology including embryonic axis specification, and cell, tissue and organ morphogenesis and
61 homeostasis [1]. Dishevelled (Dsh/Dvl) is a central integrator of the three main Wnt signaling
62 pathways - the canonical Wnt/ β -catenin pathway which drives cell specification, and the non-
63 canonical Wnt/planar cell polarity and Wnt/ Ca^{2+} pathways controlling cellular morphogenesis [2-
64 4]. Despite decades of research and clear evidence of the essential nature of Dsh in Wnt signal
65 transduction in a number of species, fundamental questions remain about how Dsh is activated,
66 regulated, and localized in cells [2,5].

67 The sea urchin embryo has proved to be an exceptional experimental model for studying
68 gene regulatory networks in general and how the canonical Wnt/ β -catenin (cWnt) pathway

69 regulates animal-vegetal axis determination in particular [6-8]. In the cWnt branch of this
70 pathway Wnt ligand binding to the LRP5/6 and Frizzled (Fz) receptor complex activates Dsh
71 which then inhibits the destruction complex made up of Axin/APC/GSK-3 β /CK1 α that targets β -
72 catenin for proteasome-mediated degradation. The escape of β -catenin from the destruction
73 complex allows for it to accumulate first in the cytoplasm and then translocate into the nucleus
74 where it acts as a transcription coactivator for a number of developmentally significant genes. In
75 the sea urchin, localized activation of cWnt signaling with its associated β -catenin nuclearization
76 in the vegetal blastomeres is a crucial determinant of endomesodermal specification and the
77 patterning of the animal-vegetal axis [9-13]. The critical role for cWnt signaling in the early
78 specification of the vegetal pole suggests that this region of the early sea urchin embryo may be
79 enriched in Wnt ligand or receptors [14]. However, none of the maternally expressed Wnts or
80 Fz receptors are preferentially expressed within the vegetal pole region of the embryo according
81 to mRNA localization [12,13,15,16]. In addition, it has been argued that β -catenin nuclearization
82 in the vegetal blastomeres is a cell autonomous process not under the influence of extracellular
83 Wnt ligands given that nuclear localization of β -catenin occurs in putative vegetal blastomeres of
84 dissociated embryos [10].

85 Interestingly, Dsh in sea urchin embryos has been shown to not only play its expected role
86 in the cWnt pathway regulation of the β -catenin-dependent gene expression of vegetal cells
87 [12,17], but also to localize in discrete puncta in a novel vegetal cortical domain (VCD) that
88 arises in oocytes and persists in eggs and embryos [12,14,17,18]. The VCD was initially
89 recognized in early embryos overexpressing Dsh-GFP [17,18] and subsequently shown to exist
90 in oocytes, eggs and embryos up to the 60-cell stage using immunofluorescent localization [14].
91 Even though Dsh is maternally expressed uniformly in the egg and early embryo [16,17], current
92 evidence indicates that the VCD region is critical for its activation and the triggering of the cWnt
93 pathway in the vegetal cells of the embryo. For example, overexpression of Dsh in zygotes had
94 no impact on embryonic development whereas physical dissection of the VCD prior to
95 fertilization resulted in abnormal, animalized/anteriorized embryos [12], an outcome also seen in
96 embryos overexpressing dominant negative Dsh [17]. In addition, transplantation of the VCD to
97 the animal pole induced the generation of ectopic endoderm [12]. All these results suggest that
98 Dsh alone is not sufficient to direct proper cWnt-based development, but that instead it needs to
99 be associated with the VCD which may act as a scaffold for localized Dsh and subsequent cWnt
100 pathway activation [14].

101 Despite the developmental importance of the VCD, relatively little is known about the precise
102 structural organization of the Dsh localized there. Past studies have shown that Dsh is

103 arranged in cortex associated puncta and mutational analysis suggests that Dsh VCD binding is
104 based on a N-terminal lipid-binding motif, the DIX domain, and a 21 amino acid motif between
105 the Dsh PDZ and DEP domains [17,18]. Previous work also suggests that the Dsh puncta are
106 not sensitive to disruption of actin filaments or microtubules in the short term, however, over the
107 longer term cytochalasin-based actin disruption led to the unexpected degradation of Dsh pools
108 in the egg [14]. Poorly-defined punctate staining patterns, indications of membrane vesicle
109 binding, and/or associations with the cytoskeleton have been reported for Dsh in cells from a
110 broad range of different species [2]. However, Dsh localization in cells remains controversial
111 given that the punctate staining patterns have been considered non-physiological due to
112 potential artifacts associated with biomolecular condensates formed due to Dsh over-expression
113 [19], and other studies have suggested that Dsh can activate the cWnt pathway in the absence
114 of cytoplasmic puncta [20].

115 In the present study we have localized sea urchin Dsh in the VCD at the ultrastructural level
116 using super-resolution immunofluorescence microscopy and immuno-gold TEM in order to
117 reveal the nanoscale architecture of plasma membrane tethered Dsh and investigate its
118 potential relationship with cortical membrane structures and the actomyosin cytoskeleton. Our
119 results indicate that Dsh in the sea urchin egg VCD is organized into well-defined puncta that
120 consist of aggregates of multiple Dsh proteins that appear as groupings of pedestal-like
121 structures interspersed between the microvillar cores of actin filaments in the cortex. These Dsh
122 patches appear associated with the membrane but are not sensitive to detergent extraction
123 suggesting an association with membrane proteins. We do not see evidence of a direct
124 interaction between Dsh and cortical actin filaments given an analysis of our images and the
125 persistence of Dsh puncta in eggs in which actin filaments have been disrupted with latrunculin
126 treatment. These results confirm and extend previous studies and reveal, for the first time in any
127 cell type, the ultrastructural organization of plasma membrane tethered Dsh.

128

129

130 **Materials and Methods**

131 **Animals, antibodies, and reagents**

132 *Lytechinus pictus* sea urchins were purchased from Marinus Scientific (Lakewood, CA) and
133 *Strongylocentrotus purpuratus* sea urchins were collected from the waters surrounding Port
134 Townsend, WA, and maintained at the Friday Harbor Laboratories (Friday Harbor, WA). All
135 animals were kept in either running natural sea water or closed artificial sea water systems at

136 10-15°C. Research on sea urchin invertebrate animals is not regulated by or subject to approval
137 from the Institutional Animal Care and Use Committee of Dickinson College.

138 Primary antibodies used included anti-SUDsh-C, an affinity-purified rabbit polyclonal
139 antibody raised against a synthetic peptide (NH₂-CMVPMMPRQLGSPEDLSGS-COOH) based
140 on a phylogenetically conserved sequence from the sea urchin Dsh protein C terminus, a
141 mouse monoclonal antibody against a highly conserved epitope of chicken gizzard actin (clone
142 C4) from EMD Millipore (Burlington, MA), and a mouse monoclonal antibody against the Ser19
143 phosphorylated form of the myosin II regulatory light chain (P-MyoRLC) from Cell Signaling
144 Technology (Danvers, MA). Appropriate secondary antibodies conjugated to Alexa Fluor 488,
145 568, or Oregon Green as well as Alexa Fluor 633 conjugated phalloidin were obtained from
146 Molecular Probes (Eugene, OR). Secondary antibody conjugated to 18 nm colloidal gold was
147 obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The actin filament
148 disruptor Latrunculin A (100 µg/ml stock in ethanol) and the myosin II light chain kinase (MLCK)
149 inhibitor ML-7 (100 mM stock in DMSO) were obtained from Cayman Chemical (Ann Arbor, MI),
150 whereas the fixable fluorescent membrane dye FM1-43FX (2 mM stock in methanol) was from
151 Molecular Probes. Unless otherwise indicated, the majority of other reagents were purchased
152 from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

153

154 **Gamete collection, fertilization, cortex isolation, and inhibitor treatments**

155 Sea urchin gametes were collected via intracoelomic injection with 0.5 M KCl, with sperm
156 collected dry and eggs spawned in either natural sea water or MBL artificial sea water (ASW:
157 423 mM NaCl, 9 mM KCl, 9.27 mM CaCl₂, 22.94 mM MgCl₂, 25.5 mM MgSO₄, 2.14 mM
158 NaHCO₃, pH 8.0) and subsequently dejellied by multiple washes with ASW. Eggs were fertilized
159 by addition of dilute sperm, the fertilization envelopes removed using 1 M urea (pH 8.0), and
160 then washed into and reared in MBL calcium free sea water (CFSW: MBL ASW minus CaCl₂
161 and plus 1 mM EGTA) at 10-15°C.

162 Cortices of unfertilized eggs and first division cycle embryos were generated as described in
163 [21]. In brief, eggs/embryos were allowed to quickly settle onto poly-L-lysine (2 mg/ml) coated
164 coverslips and then exposed to fluid shear force from a pipette containing an isotonic cortex
165 isolation buffer (CIB: 0.8 M mannitol, 5 mM MgCl₂, 10 mM EGTA, 100 mM HEPES, pH 6.8 for
166 unfertilized eggs and pH 7.4 for embryos). Isolated cortices were rinsed twice in CIB prior to
167 further processing for light microscopic fluorescence localization.

168 In order to test the impact of detergent extraction on Dsh localization, isolated cortices were
169 treated with 1% Triton X-100 in CIB for one minute following isolation and immediately prior to

170 fixation. For disruption of actin filaments, eggs in ASW were treated for 20 min with 1 μ M
171 Latrunculin A. The effect of the inhibition of myosin II light chain kinase (MLCK) was tested by
172 treating eggs in ASW for 20 min with 50 μ M of the MLCK inhibitor ML-7.

173

174 **Fixation, fluorescent staining and light microscopic imaging and analysis**

175 Isolated cortices plus and minus Triton extraction were fluorescently stained for membranes
176 using 1-2 μ M FM1-43 [22] for two minutes prior to fixation. Cortices were fixed in 2-4%
177 formaldehyde in CIB for 15 min followed by blocking in 2% goat serum and 1% BSA in PBS for
178 30 minutes. Immunostaining was performed with appropriate primary and secondary antibodies
179 diluted in the range of 1:200 to 1:300 in blocking buffer and staining took place for 30-60 min for
180 each stage. Fluorescent phalloidin was added to the secondary antibody staining step. Cortex
181 samples for conventional and 3D-SIM microscopy were mounted in nonhardening Vectashield
182 antifade mounting media (Vector Laboratories, Burlingame, CA), whereas STED imaging
183 samples were mounted in Prolong Diamond mounting media (Molecular Probes).

184 Wide-field epifluorescence microscopy of samples was performed on a Nikon (Tokyo,
185 Japan) 80i microscope using either a 40X/0.75 NA Plan Fluor (phase contrast or DIC) or
186 60X/1.4 NA Plan Apo phase contrast objective lens with digital images captured using a
187 Photometrics (Tucson, AZ) CoolSnap Cf cooled CCD camera. Super-resolution microscopy
188 was performed using two different methods. For 3D structured illumination microscopy (3D-SIM,
189 [23] we utilized a DeltaVision OMX 3D-SIM Imaging System (GE Healthcare Bio-Sciences,
190 Pittsburgh, PA) with an Olympus 60X/1.42 NA objective lens. Captured images were
191 reconstructed using SoftWoRx software. Stimulated Emission Depletion (STED) super-
192 resolution microscopy [24] was performed on a Leica (Wetzlar, Germany) Sp8 STED confocal
193 using a 100X/1.4 NA objective lens. For all forms of microscopic images, processing and
194 analysis was performed using Fiji/ImageJ (Bethesda, MD). Graphs were prepared and statistical
195 analysis carried out using Graphpad Prism 8 (San Diego, CA) and figures prepared using
196 Adobe Photoshop (San Jose, CA).

197

198 **Immuno-EM localization and platinum replica TEM**

199 Immuno-EM localization of Dsh followed the methods of Svitkina [25,26]. Isolated egg
200 cortices were fixed with 0.25% glutaraldehyde in CIB for 5 min, rinsed with PBS and then
201 quenched for 10 min in 2 mg/ml NaBH₄ in PBS. Following blocking in 2% goat serum, 1% BSA,
202 and 1 mg/ml glycine in PBS for 30 min cortices were incubated in primary anti-SUDsh-C
203 antibody for 60 min followed by overnight incubation in colloidal gold-conjugated secondary

204 antibody in immunogold buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.05% Tween 20, 0.1%
205 BSA). Following rinses in immunogold buffer, cortices were post fixed with 2% EM-grade
206 glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3 for 30 min.

207 The generation of critical point-dried and rotary-shadowed platinum replicas of
208 immunolabeled isolated cortices followed previously described methods [25-27]. Briefly, post
209 fixation cortices were treated with aqueous 0.1% tannic acid followed by aqueous 0.2% uranyl
210 acetate. Then the samples were dehydrated in a graded ethanol series, critical point dried, and
211 rotary shadowed with platinum and carbon. The platinum replicas of cortices were separated
212 from the glass coverslips using hydrofluoric acid, mounted on Formvar-coated grids, and
213 observed on a JEM 1011 TEM (JEOL, Peabody, MA) operated at 100 kV and digital images
214 captured with an ORIUS 832.10W CCD camera (Gatan Inc, Warrendale, PA) and presented in
215 inverted contrast. All EM reagents and materials were obtained from Electron Microscopy
216 Sciences (Hatfield, PA).

217

218

219 **Results and Discussion**

220 **The Dsh array in the VCD as visualized with super-resolution microscopy of isolated egg** 221 **cortices**

222 Super-resolution imaging using 3D-SIM showed that the Dsh localization pattern in the VCD
223 of isolated egg cortices appeared similar to a dot diagram of a concentration gradient (Fig 1A-
224 H), as was previously documented using confocal imaging [14]. In the center of the VCD the
225 density of Dsh puncta is high and this density diminished when moving toward the edge of the
226 distribution (Fig 1I). Measurements of the Dsh density per square micron ($n = 15$ cortices over
227 3 separate experiments) varied from an average of 3.6 in the center, to 1.9 midway in the
228 distribution, to 0.7 in the sparse region of the edge (Fig 1I,J), with the differences in density
229 between these three regions being statistically significant ($p < 0.001$ based on a one-way
230 ANOVA). 3D-SIM imaging (Fig 1A-H) suggested that the Dsh puncta were discrete structures.
231 However, imaging with higher resolution STED microscopy revealed that some of the Dsh
232 puncta seen as individual fluorescent dots in confocal imaging (Fig 1M, arrows) were resolved
233 as being composed of multiple spots in the STED images (Fig 1L, arrows). This indicated that
234 the Dsh puncta consisted of aggregates of multiple Dsh proteins. The resolving power of STED
235 is generally considered to be two-fold higher than SIM which is itself two-fold higher than
236 conventional confocal imaging [28].

237 **Fig 1. Super-resolution imaging of Dsh and actin staining in the VCD in isolated egg cortices.** (A-
238 H) 3D-SIM imaging of Dsh (magenta) and F-actin (green) staining of cortices isolated from *L. pictus* (A-D)
239 and *S. purpuratus* (E-H) eggs showing the distribution of Dsh puncta relative to microvillar core actin foci.
240 D and H are higher magnification versions of the white boxes in C and G, and elongate submembranous
241 actin filaments are visible in D (arrow). (I-M) The distribution of Dsh in the VCD appears similar to a
242 diagrammatic representation of a concentration gradient. As shown with the enlarged versions of the red
243 boxes in I, the lowest density of Dsh puncta are at the VCD edge, with increased density in the midway
244 point, and the maximum density in the center. (J) Quantification of Dsh densities in the three regions of
245 the VCD. The Dsh VCDs of 5 cortices each from three separate experiments were analyzed and the
246 densities in these 3 regions are all statistically significantly different. (K-M) STED imaging of Dsh staining
247 in the *S. purpuratus* egg VCD reveals that single punctum in confocal images (L, arrows) often appear as
248 multiple spots in the higher resolution STED images (M, arrows). Scale bars = 5 μm .

249
250 Within the VCD the Dsh puncta were interspersed with actin filaments that were present in
251 two basic organizations: bright foci representing filament aggregations within the cores of the
252 short microvillar found on the surface of unfertilized eggs [29,30], and, particularly in *L. pictus*
253 cortices, faint long actin filaments running parallel to the plane of the plasma membrane (Fig 1D
254 arrow). The more elongate microvillar actin staining pattern seen in the *L. pictus* egg cortices
255 (Fig 1A-D) relative to the more circular microvilli in *S. purpuratus* cortices (Fig 1E-H) are
256 indicative of species-specific differences in microvillar morphology [30]. Our 3D-SIM images did
257 not suggest a direct interaction between microvillar actin arrays and Dsh puncta, although in
258 other cells and tissues it has been suggested that Dsh interacts with actin filaments, particularly
259 within stress fibers and focal adhesions [31,32].

260
261 **Platinum replica TEM of the egg VCD indicates that Dsh puncta consist of aggregates of**
262 **pedestal-like structures**

263 Immunogold labeling combined with platinum replica TEM of isolated cortices has allowed us
264 to investigate the ultrastructure of the Dsh puncta visualized at the light microscopic level. Low
265 magnification TEM images of cortices labeled for Dsh with 18 nm colloidal gold (colored gold in
266 Fig 2A-D; Fig 3A-H) demonstrated a sparse array of gold decorated aggregates (arrows in Fig
267 2A) amongst a distribution of microvillar core (MV in Fig 2B) and submembranous actin
268 filaments, as well as the remnants of membranous cortical granules (CG in Fig 2A), vesicles
269 and endoplasmic reticulum. Higher magnification imaging allowed for the identification of actin
270 filaments (colored green in Fig 2E) due to their characteristic platinum replica TEM appearance
271 [25-27,33] and revealed that Dsh labeled aggregates appeared in patches on the cytoplasmic

272 face of the plasma membrane (colored magenta in Fig 2E). A gallery of Dsh-labeled aggregates
273 (Fig 3A-F) showed that single colloidal gold particles were located on the top of pedestal-
274 shaped structures that grouped together into patches that were distinct from the surrounding
275 plasma membrane and underlying meshwork of the vitelline envelope. Within the aggregates
276 the labeled structures often appeared close together and on occasion adopted linear or ring-
277 shaped arrangements (Fig 3E,F). The Dsh aggregates did not colocalize with the tangles of
278 short actin filaments present in microvillar cores (Figs 2 and 3), however they did on occasion
279 show an association with submembranous elongate actin filaments running parallel with the
280 plane of the membrane (Figs 2E,F and 3A,C,F,I). In terms of quantitative analysis of TEM
281 images, the density of the distribution of Dsh aggregates in the TEM images fell within the range
282 of those seen with light microscopy (Fig 3G), and the overall average area of single Dsh labeled
283 aggregates was $\sim 18,200 \text{ nm}^2$ (Fig 3H; $n = 5$ cortices over 2 separate experiments). Comparison
284 of regions of the same cortex that contained Dsh-labeled patches (Fig 3I) with areas that did not
285 (Fig 3J) revealed that these unlabeled regions did not contain similar membrane-associated
286 structures (Fig 3J), indicating that these structures were specific to the Dsh puncta in the VCD.
287

288 **Fig 2. Platinum replica TEM of Dsh and actin in the VCD in isolated egg cortices.** Low (A, C) to
289 medium (B,D) magnification images of cortices shows Dsh-specific colloidal gold (colored gold) staining of
290 patches in the plane of the membrane (arrows in A). Tangled knots of short actin filaments appear in the
291 cores of microvilli (MV in B) and numerous elongate actin filaments running parallel to the plane of the
292 membrane are present. Cortical granules appear as shriveled structures (CG in A) and other
293 membranous structures are also present. The meshwork that appears in the background of the images
294 corresponds to the vitelline envelope. The white box in C appears at higher magnification in E in which
295 Dsh aggregates are colored magenta and identifiable actin filaments in green. Dsh positive patches do
296 not associate with MV core actin assemblages but do come in close proximity to submembranous actin
297 filaments. (F,G) High magnification images indicate that Dsh patches consist of aggregates of pedestal-
298 like structures - each labeled with a single colloidal gold particle - that can be grouped into one or more
299 clusters. *L. pictus* cortices = A,B,F,G; *S. purpuratus* cortices = C,D,E. Bar length indicated in the images.
300

301 **Fig 3. Platinum replica TEM demonstrates that Dsh puncta correspond to patches of aggregates of**
302 **pedestal-like structures.** (A-F) A gallery of high magnification images of Dsh labeled structures
303 demonstrates that they consist of aggregates of pedestal-like structures in a variety of groupings. The top
304 of each pedestal is labeled with a single colloidal gold particle suggesting that this area may correspond
305 to the location of the C terminus of the Dsh protein. (G) The density of the Dsh puncta in TEM images
306 from 3 separate cortices over three experiments (grey box) falls in the range of densities seen in the
307 immunofluorescence images (black boxes reproduced from graph Fig 1J). (H) The area of the Dsh

308 aggregates from five cortices each from 2 separate experiments shows an average of ~18,200 nm², and
309 the differences in the areas between the two species are not statistically significant. (I, J) Comparison of
310 regions of the same cortex in which Dsh labeling is present (I) and is not present (J) shows that the
311 patches associated with the Dsh labeling are not visible in the unlabeled regions, suggesting that these
312 structures are specific to the Dsh puncta. *L. pictus* cortices = A,B,D,E; *S. purpuratus* cortices = C,F,I,J.
313 Scale bars = 200 nm.

314

315 Weitzel et al. [17] and Leonard and Etensohn [18] conducted mutational analysis of the
316 association of sea urchin Dsh-GFP with the VCD. Their results indicated that this localization
317 could be abolished by deletion of the entire N terminus, the DIX domain, a double mutation of
318 the phospholipid-binding region of DIX (K57A, E58A), or a 21 amino acid motif between the
319 PDZ and DEP domains. These results combined with our TEM images suggest that Dsh is
320 oriented with its N terminal domain closer to the membrane which may explain why our
321 SUDshC-terminus specific antibody appears to label the tops of the pedestal-like structures that
322 are located farther away from the membrane surface. Dsh protein has also been shown to form
323 oligomers via interactions between DIX domains in vitro and in vivo [34] and it is possible that
324 the Dsh aggregates seen in our TEM images may represent some form of Dsh oligomerization
325 in association with the membrane.

326

327 **The Dsh VCD array is resistant to Triton detergent extraction, disruption of actin** 328 **filaments, and inhibition of myosin II contraction**

329 The punctate Dsh staining present in sea urchin eggs and embryos and in many other cell
330 types has been hypothesized to be caused by an association of Dsh with intracellular vesicles
331 [2,17,18]. We tested the nature of the interaction between the Dsh puncta and membranes by
332 performing a 1% Triton X-100 detergent extraction of egg cortices immediately after isolation
333 and prior to fixation. Dsh immunofluorescent staining of the VCD persisted following detergent
334 extraction (Fig 4A-H) suggesting that the Dsh is in a detergent resistant structure associated
335 with the plasma membrane. The disruption of membranes by the Triton-based extraction of
336 cortices was confirmed by the absence of cortical granules in phase contrast images of
337 extracted cortices (Fig 4D,H), as well as the loss of the membrane-dependent fluorescent
338 staining with the lipophilic dye FM1-43 (Fig 4I-N). These results suggest that Dsh interacts with
339 either detergent resistant regions of the membrane – such as lipid rafts which have been
340 previously identified in sea urchin eggs [35] – and/or it may be binding to a detergent resistant
341 membrane protein assemblage. This lack of detergent sensitivity argues against the association
342 of Dsh with the membranes of cortex-associated vesicles in the VCD, which is agreement with

343 studies in other cells suggesting that Dsh puncta are not associated with cytoplasmic vesicles
344 [36]. This includes work in *Xenopus* oocytes and embryos in which vegetal pole-associated Dsh
345 puncta are argued to help direct cWnt-dependent axis specification following cortical rotation
346 [37,38]. Tadjuidje et al. [39] have reported that *Xenopus* oocytes depleted of maternal Dsh
347 (Dvl2/3) RNA still can activate cWnt signaling and that this derives from the persistence of Dsh
348 puncta in the submembranous region of the vegetal cortex. These Dsh puncta did not
349 codistribute with markers for endosomes, exocytotic vesicles or lysosomes and therefore do not
350 appear associated with vesicles and may instead correspond to protein aggregates [39].

351
352 **Fig 4. The Dsh VCD array is resistant to Triton detergent extraction.** (A-H) Control isolated egg
353 cortex from *L. pictus* (A-D) stained for Dsh (magenta) and actin (green) showing Dsh array and cortical
354 granules in phase contrast. The Triton extracted cortex (E-H) demonstrates the persistence of the Dsh
355 array following detergent extraction despite the loss of cortical granules seen in phase contrast. (I-N)
356 Membrane staining with the fixable dye FM1-43 (green) shows that in control egg cortices (I-K) that the
357 Dsh (magenta) array is present along with a variety of membranous structures. In detergent extracted
358 cortices (L-N) the Dsh array is still present even though specific membrane staining is lost. Scale bars =
359 10 μ m.

360
361 Earlier work by [14] has indicated that the Dsh VCD localization in the sea urchin egg is
362 sensitive to disruption of actin filaments using the drugs cytochalasin B and D and >2 hour
363 incubation periods following a 20 min drug treatment. In addition, studies in other cell types
364 have argued for an interaction between Dsh and actin filaments [31,32]. We tested the
365 association between actin and Dsh in the short term using the drug latrunculin A (LatA). Unlike
366 the cytochalasins that tend to interfere with actin monomer addition to the plus end, LatA
367 disrupts actin filaments primarily via monomer binding and subsequent sequestration. We
368 treated embryos with 1 μ M LatA for 20 min followed by isolation of cortices and anti-Dsh
369 immunofluorescent staining. Dsh labeling of the VCD was maintained in LatA treated egg
370 cortices even though the LatA led to a clear disruption of cortical actin filaments (Fig 5D-F). We
371 also tested for the potential involvement of myosin II in the Dsh VCD localization given that the
372 actomyosin cortex has been shown to influence the asymmetric distribution of the important
373 polarity determining PAR protein complex in *C. elegans* [40,41] and sea urchin [42] embryos. In
374 sea urchins, the apical PAR localization was disrupted by treating embryos with the MLCK
375 inhibitor ML-7 [42]. MLCK inhibition is known to block myosin II bipolar filament assembly and
376 the enhancement of actin activated ATPase activity, and ML-7 has been used extensively as a
377 myosin II inhibitor in previous studies on sea urchin embryos [42-45]. We treated eggs with 50

378 μM ML-7 for 20 min and then isolated the cortices and stained for Dsh. ML-7 treatment had no
379 impact on Dsh VCD localization or actin distribution in the egg cortices (Fig 5G-I).

380

381 **Fig 5. The Dsh VCD persists following disruption of actin filaments or inhibition of myosin II**
382 **contraction.** Control isolated egg cortex from *L. pictus* (A-C) contains the expected Dsh array (magenta)
383 along with microvillar actin (green). In cortices from eggs treated with the actin filament disrupting drug
384 LatA (D-F) the Dsh array persists (D) whereas the actin staining is greatly reduced (E). Both Dsh (G) and
385 actin (H) staining appear unaffected in cortices isolated from eggs treated with the MLCK inhibitor ML-7
386 (G-I) in order to inhibit myosin II contraction. Scale bar = 10 μm ; magnifications of A-I are equivalent.

387

388 **The Dsh VCD array is bisected by the cytokinetic contractile ring in isolated cortices of**
389 **first division embryos**

390 The Dsh VCD array persists post-fertilization [14,17,18] despite the extensive remodeling of
391 the plasma membrane caused by the mass exocytosis of cortical granules followed by the
392 endocytic uptake of membrane [46]. We investigated the behavior of the Dsh VCD array in early
393 cleavage embryos by labeling cortices isolated from first division embryos with Dsh and probes
394 specific for the cytokinetic contractile ring proteins myosin II and actin. We were interested in the
395 relationship between the Dsh VCD array and the contractile ring given that our previous work
396 [27] has indicated that the contractile ring consists of a very dense assemblage of actin and
397 myosin II filaments in close association with the plasma membrane. Previous work with either
398 Dsh-GFP [17] or Dsh immunofluorescent labeling of whole embryos [14] has suggested that the
399 first division cleavage furrow bisects the Dsh VCD. Cortices stained for Dsh and activated
400 myosin II via staining with an antibody against the Ser19 phosphorylated version of the myosin
401 II regulatory light chain revealed that the Dsh array coexisted with the contractile ring in regions
402 in which they are both found (Fig 6). In the majority of isolated cleavage cortices, the Dsh
403 distribution appeared to be bisected by the contractile ring structure (Fig 6), as would be
404 expected for the two daughter cells to inherit roughly equivalent amounts of the VCD. The Dsh
405 VCD array in first division cortices is not as highly ordered as the VCD in egg cortices given that
406 it often does not closely resemble a concentration gradient pattern and the individual puncta
407 appear larger and less densely distributed (Fig 6).

408

409 **Fig 6. The Dsh VCD in cortices isolated from first division embryos is bisected by the cytokinetic**
410 **contractile ring.** (A-Q) Widefield image of cortices isolated from first division *S. purpuratus* embryos
411 indicate that the Dsh punctate VCD array (A,E,I,N; magenta in C,G,L,Q) is often bisected by the
412 cytokinetic contractile ring which labels for activated myosin II (B,F,J,O; green in C,G,L,Q) and F-actin

413 (K,P; blue in L,Q). The Dsh VCD does not appear as well ordered as the arrays in egg cortices (Fig 1),
414 and individual puncta appear larger and less numerous. Transmitted light DIC (D,H at equivalent
415 magnification) and phase contrast (insets M,R at reduced magnification) images of individual cortices are
416 provided for context. Scale bar = 10 μ m, magnifications of A-L and N-Q are all equivalent.

417

418 **Conclusions**

419 Despite some 25 years of research effort, aspects of the activation, regulation and
420 localization of the central cWnt pathway protein Dsh remain enigmatic [2-4]. In the present study
421 we use super-resolution and platinum replica transmission electron microscopy imaging of the
422 sea urchin VCD to provide the first visualization of the nanoscale structural organization of
423 membrane tethered Dsh. We show that Dsh is found associated with pedestal-like structures
424 organized into aggregates located in the plane of the membrane, and that these clusters are
425 resistant to detergent extraction and to treatment with inhibitors of actin polymerization and
426 myosin II contraction. We also demonstrate that the Dsh VCD punctate arrays codistribute with
427 the dense filamentous structure of the cytokinetic contractile ring in first division embryos. The
428 general structural organization of membrane tethered Dsh that we have characterized in the sea
429 urchin VCD may be applicable to the plasma membrane-associated Dsh puncta apparent in
430 many cell types and particularly to those found in the vegetal cortex of *Xenopus* oocytes.

431 We speculate that the Dsh labeling in the sea urchin VCD could correspond to oligomers of
432 Dsh present in the puncta and that our anti-SUDsh-C terminus antibody is labeling the end of
433 the protein that is farthest from the surface of the membrane. Another intriguing possibility is
434 that the Dsh aggregates might represent biomolecular condensates that have become
435 associated with the membrane surface, as membranes have been argued to be one of the
436 control centers of phase separation in a number of cell types [47] and condensates have been
437 argued to play important roles in cWnt signaling [48]. The major pathway proteins Dsh, Axin,
438 and APC all contain the condensate-requiring intrinsically disordered regions and have been
439 shown to form dynamic, non-membrane enclosed puncta in cells, which suggests that the β -
440 catenin destruction complex and the Dsh-dependent signalosome correspond to different forms
441 of related biomolecular condensates [48]. It is intriguing that in sea urchins [14] and in *Xenopus*
442 [37] Dsh associated with vegetal puncta undergo postranslational modifications that correlate
443 with cWnt activation. Our current working hypothesis is that the Dsh structures we have defined
444 serve as autonomous scaffolds for localized cWnt pathway activation at the vegetal pole during
445 early sea urchin development.

446

447

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457

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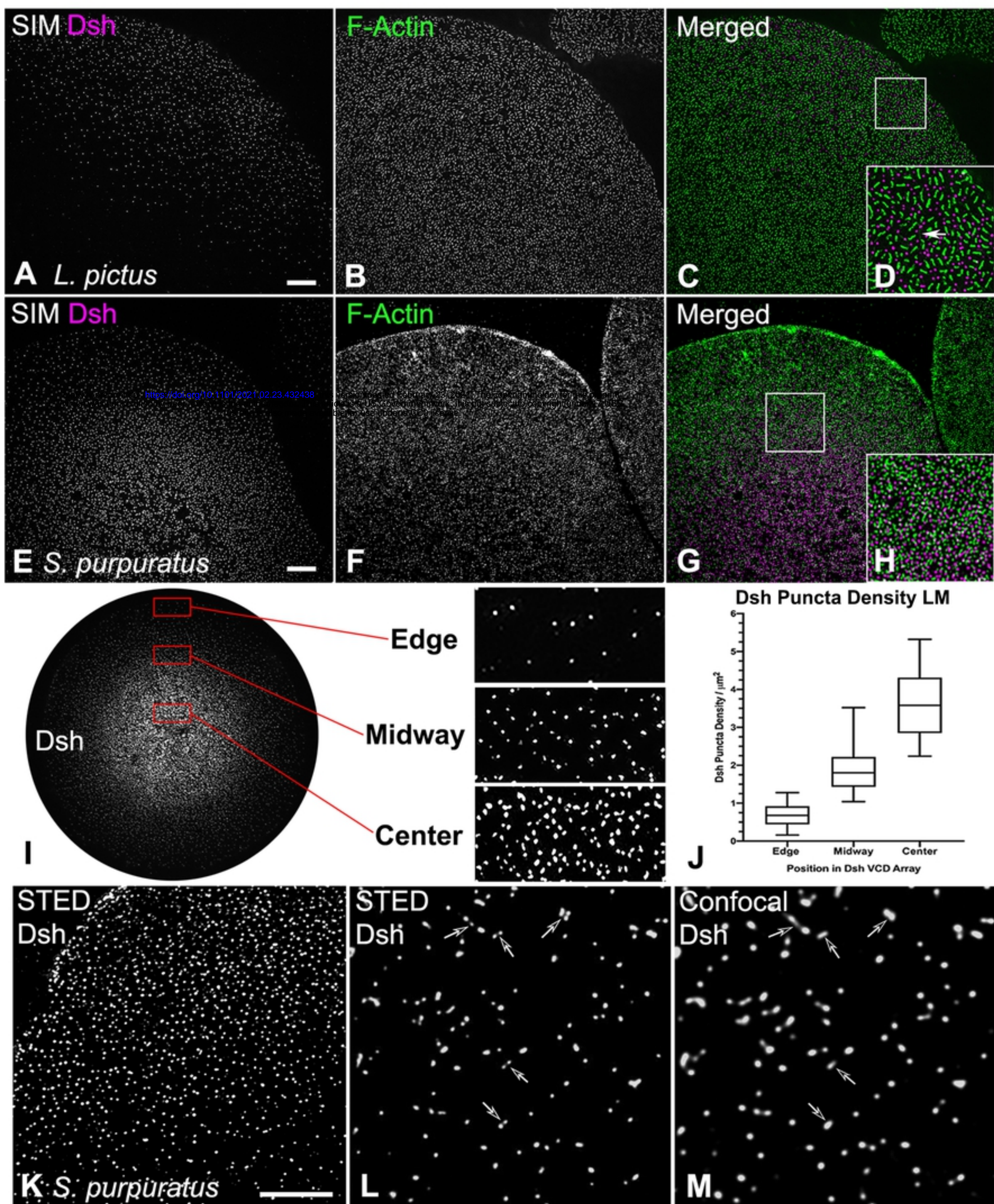


Figure 1

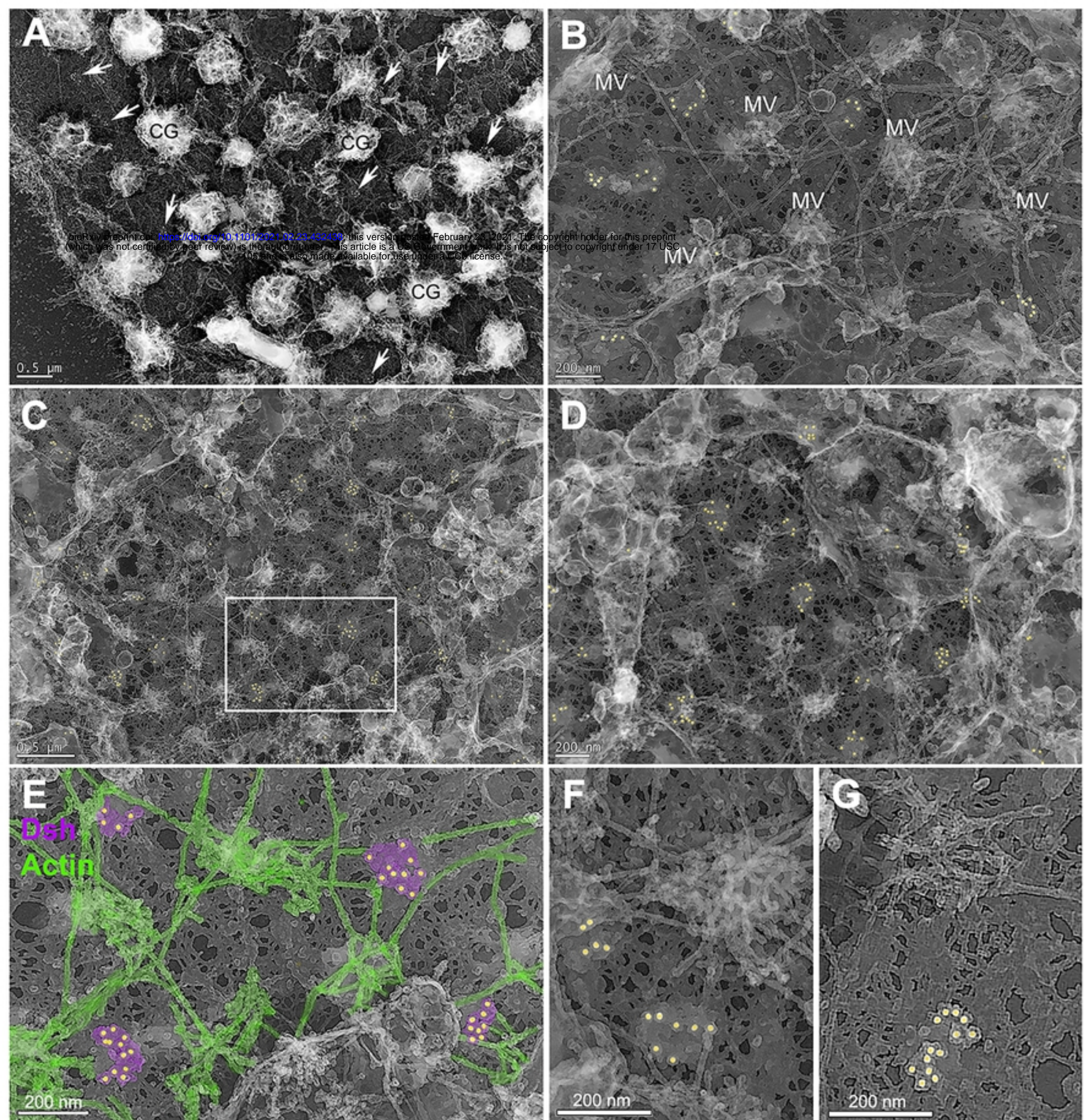
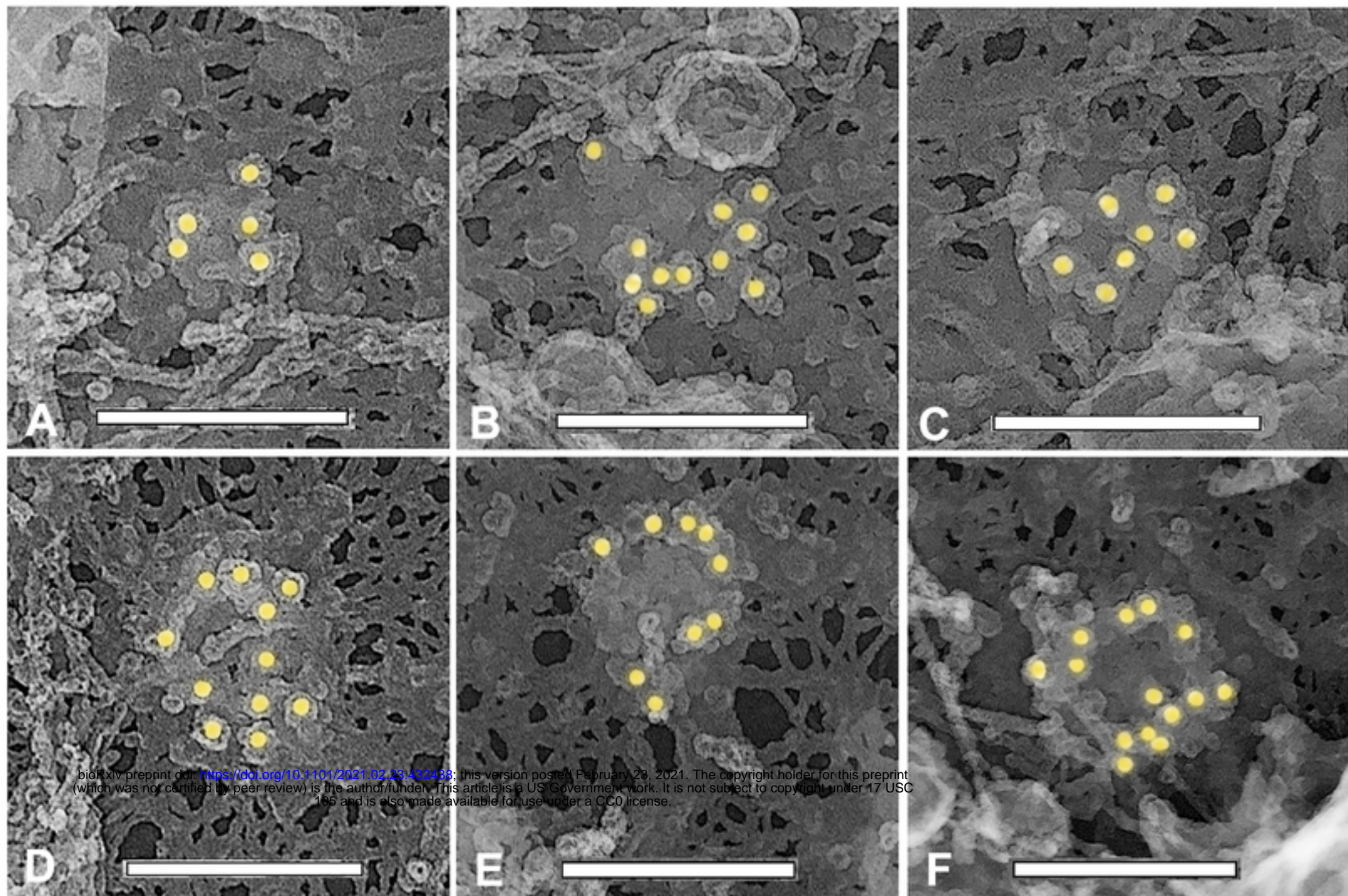
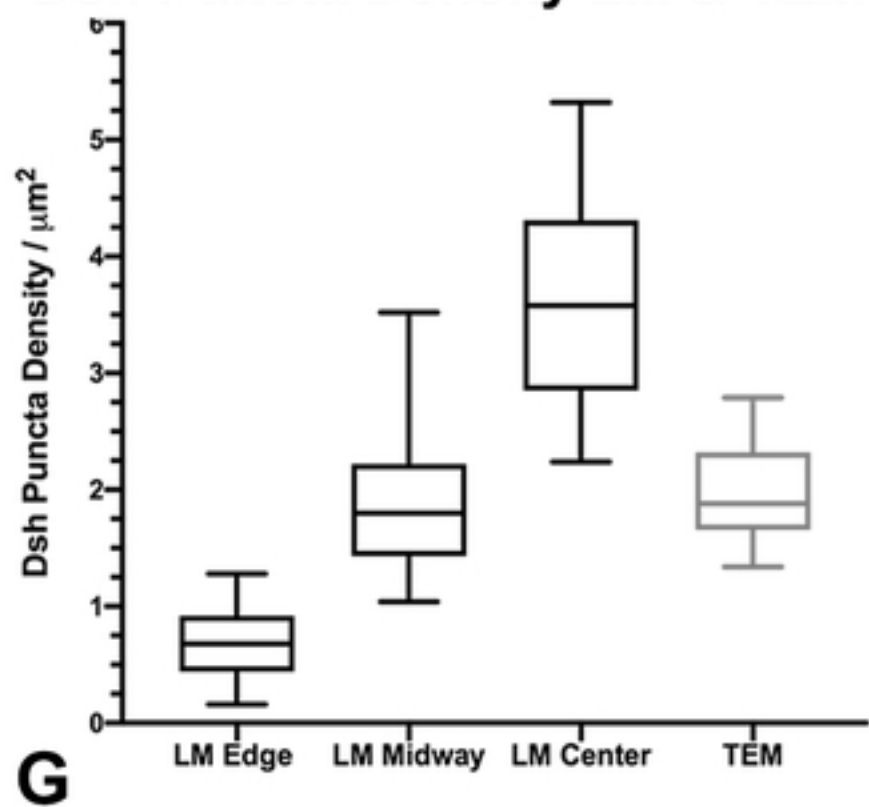


Figure 2



Dsh Puncta Density LM & TEM



Dsh Aggregate Area TEM

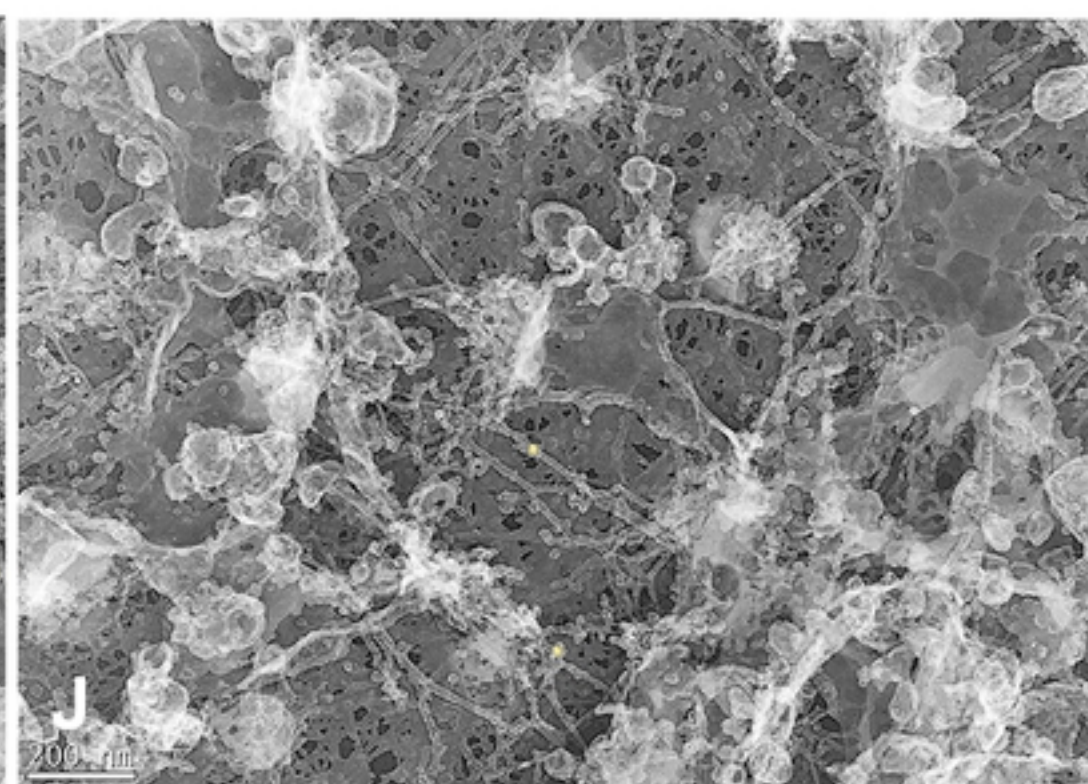
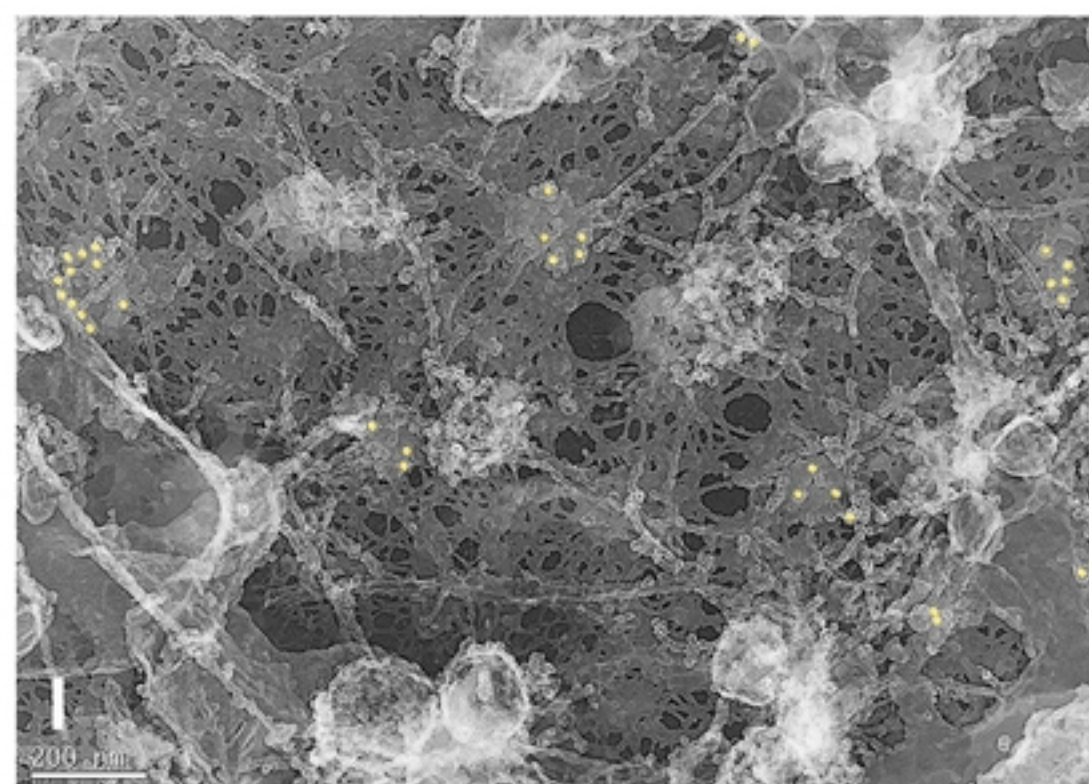
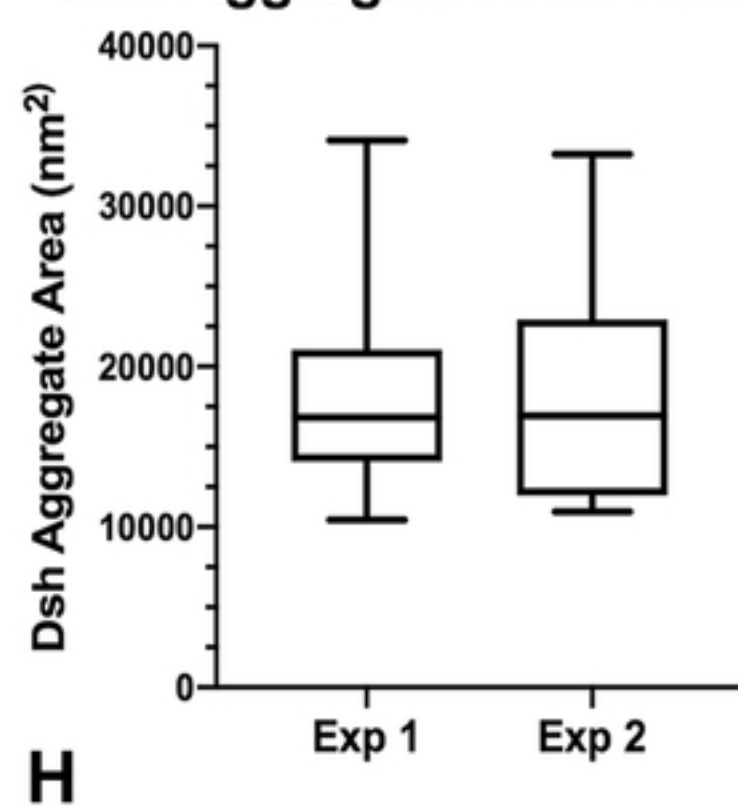
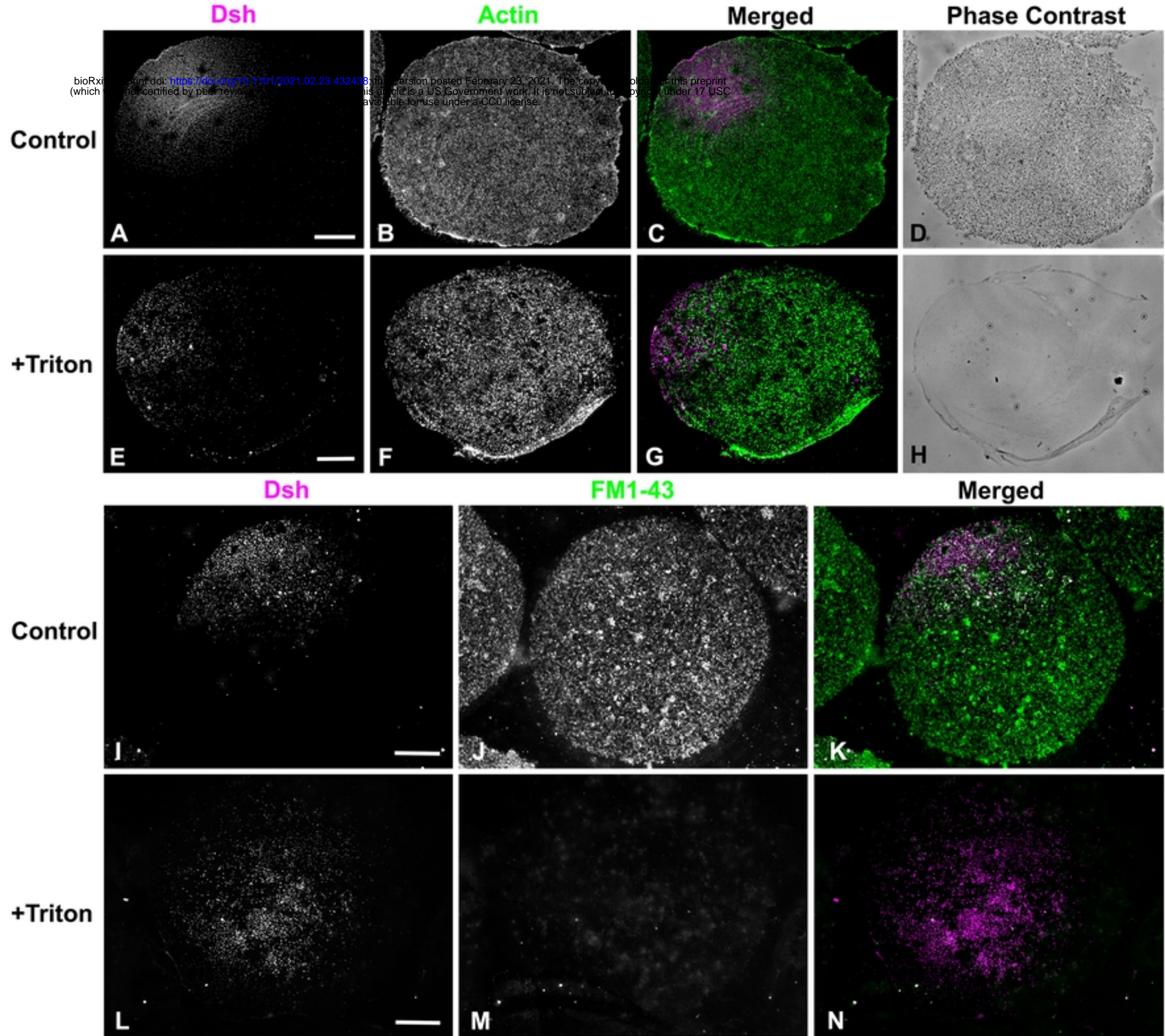


Figure 3



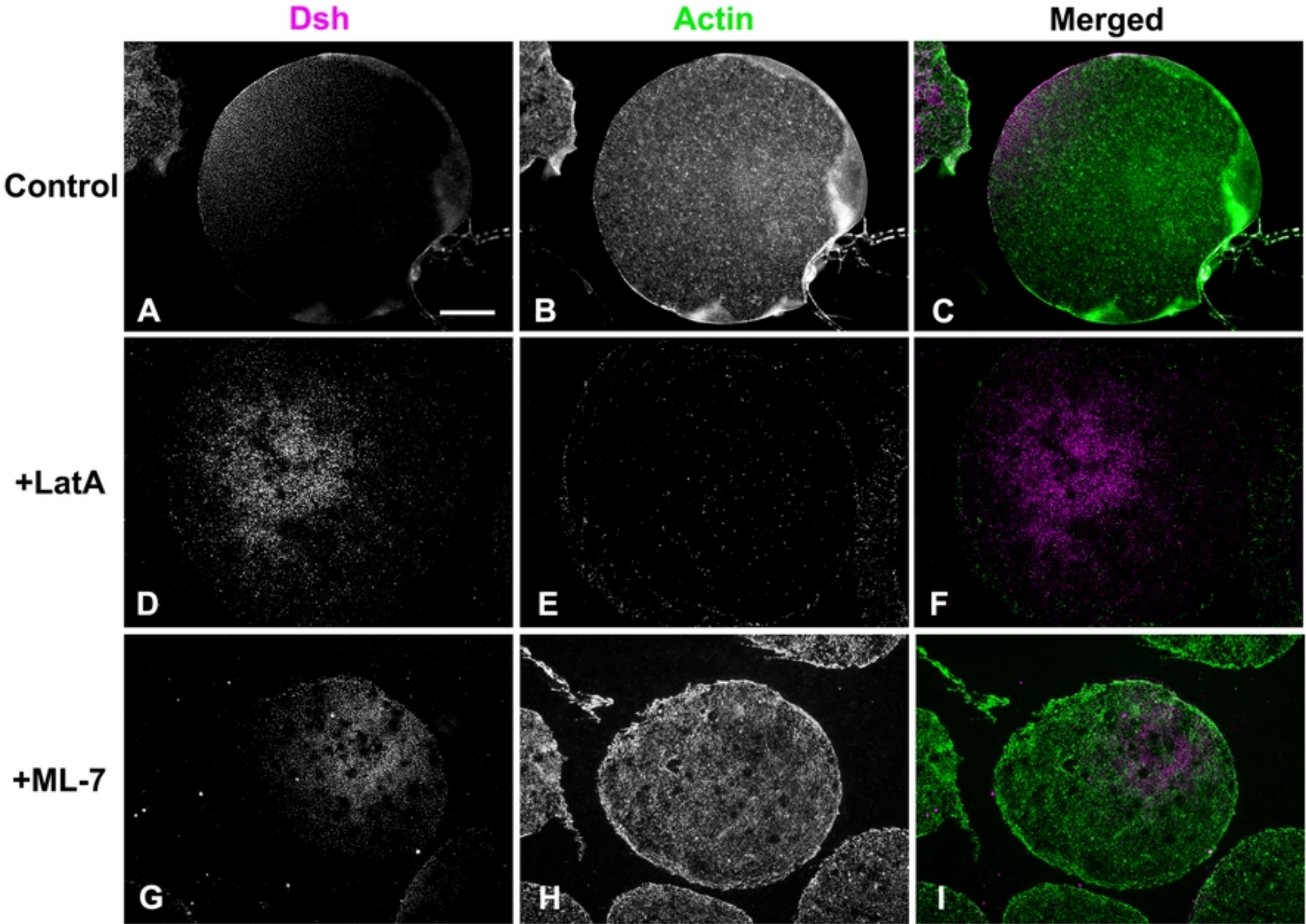


Figure 5

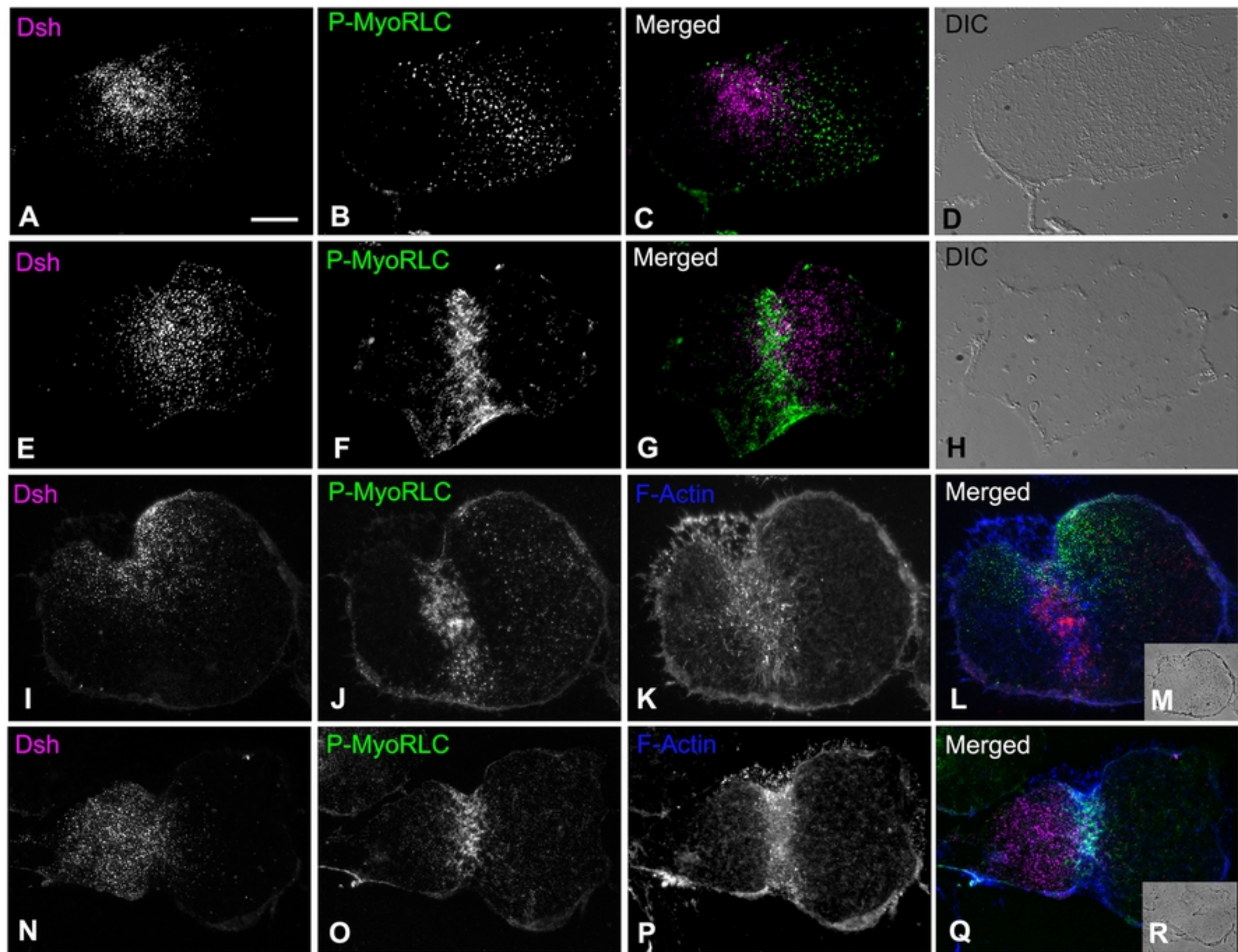


Figure 6