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2 **Acute decrease in plasma membrane tension induces macropinocytosis**

3 ***via* PLD2 activation**

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18 Running title: Membrane flaccidity induces macropinocytosis

1 **Summary**

2 We reveal a mechanical induction of macropinocytosis that is elicited by
3 acute decrease of plasma membrane tension, followed by lipid raft
4 destabilization, PLD2 activation and PA production.

6 **Abstract**

7 Internalization of macromolecules and membrane into cells through
8 endocytosis is critical for cellular growth, signaling, and membrane tension
9 homeostasis. Although endocytosis is responsive to both biochemical and
10 physical stimuli, how physical cues modulate endocytic pathways is less
11 understood. In contrary to the accumulating discoveries on effects of increased
12 membrane tension on endocytosis, little is known about how a drop of tension
13 impacts membrane trafficking. Here we reveal that acute reduction of plasma
14 membrane tension results in phosphatidic acid, F-actin and dynamin 2-enriched
15 dorsal membrane ruffling and subsequent macropinocytosis in myoblast. The
16 membrane flaccidity-induced local phosphatidic acid production depends on
17 phospholipase D2 (PLD2) that is activated *via* lipid raft disruption. Furthermore,
18 the “membrane flaccidity-PLD2-macropinocytosis” pathway is dominant in
19 myotube, reflecting a potential mechanism of membrane tension homeostasis
20 upon intensive muscle stretching and relaxation. Together, we identify a new
21 mechanotransduction pathway which converts acute tension drop into PA
22 production and subsequently initiates macropinocytosis *via* actin and dynamin
23 activities.

25 **Introduction**

26 Eukaryotic cells harness multiple endocytic pathways to internalize fluid and

1 membrane into transport vesicles from the plasma membrane (Conner and
2 Schmid, 2003; Doherty and McMahon, 2009). In response to various cellular
3 demands and environmental stimuli, endocytic machineries are regulated
4 biochemically or physically to govern the growth and survival of cells (Dai and
5 Sheetz, 1995; Liu et al., 2017; Scita and Di Fiore, 2010). Among them,
6 macropinocytosis is an endocytic pathway that is induced by biochemical
7 stimuli, including nutrients, growth factors, integrin substrates or even viruses
8 and forms actin-based membrane ruffling and engulfment that leads to the
9 formation of macropinosomes (Buckley and King, 2017; Doherty and McMahon,
10 2009).

11 Upon hyper-stimulation of growth factor receptors, activated PI3K and small
12 GTPases initiate macropinocytosis *via* phosphatidylinositol (3,4,5)-
13 trisphosphate (PIP₃) production and actin polymerization (Levin et al., 2015;
14 Yoshida et al., 2018). After membrane ruffling and closure, a sealed and large
15 endocytic vacuole (1-10 μ m in diameter) is formed. With the relatively large
16 amount of solutes and membrane area being internalized, macropinocytosis is
17 an efficient and ideal route to quickly uptake nutrients or attenuate cell signaling
18 (Doherty and McMahon, 2009). Despite the clearly-defined, biochemically
19 induced mechanism of macropinocytosis, little is known about the effects of
20 mechanical alteration on macropinosome formation.

21 Membrane tension, the force applied on plasma membrane, has emerged as
22 a master integrator which governs distinct cellular processes, including
23 membrane trafficking, cell migration, immunological responses, cell growth and
24 differentiation (Dai and Sheetz, 1995; Diz-Munoz et al., 2013; Masters et al.,
25 2013). For endocytosis, cells need to overcome higher energetic barrier to bend
26 the membrane inwardly when membrane tension is high, e.g. by using the

1 pulling force from actin polymerization in clathrin-mediated endocytosis.
 2 (Boulant et al., 2011; Tan et al., 2015; Weinberg and Drubin, 2012). Conversely,
 3 when tension is decreased, it is easier for endocytic machinery to deform the
 4 membrane thus endocytosis is increased (Saleem et al., 2015; Shi and
 5 Baumgart, 2015).

6 Plasma membrane tension is contributed by membrane-cytoskeleton
 7 adhesion and osmotic pressure (Gauthier et al., 2012). Although membrane
 8 tension is used to control multiple cellular events, cells also need to maintain
 9 the homeostasis of membrane tension by sensing the change of tension and
 10 modulating its membrane area or cytoskeletal attachment *via* endocytosis,
 11 exocytosis, membrane invagination, actin polymerization or depolymerization
 12 (Diz-Munoz et al., 2013; Gauthier et al., 2012; Nassoy and Lamaze, 2012).
 13 Together, a feedback regulation of membrane tension, actin polymerization and
 14 membrane trafficking underpins the tension homeostasis and membrane
 15 remodeling events in cells.

16 The sensors of membrane tension are generally transmembrane proteins or
 17 peripheral membrane proteins, including stretch-activated ion channels,
 18 caveolin-cavin complex, curvature sensing proteins or membrane-actin
 19 interacting proteins (Diz-Munoz et al., 2013; Tsujita et al., 2015). Recently,
 20 Petersen *et al.* reported that lipid raft could function as a mechanosensor that
 21 undergoes kinetic or mechanical disruption to activate phospholipase D2 (PLD2)
 22 (Petersen et al., 2016). PLD2 is a plasma membrane-localized lipase which
 23 catalyzes the conversion of phosphatidylcholine (PC) into phosphatidic acid (PA)
 24 and choline, and is involved in endocytosis and actin polymerization (Antonescu
 25 et al., 2010; Colley et al., 1997; Du et al., 2004; Jiang et al., 2016). PLD2 mainly
 26 localizes at a lipid microdomain, the lipid raft, on plasma membrane *via*

1 palmitoylation on its C223 and C224 residues where PC and phosphatidyl-4,5-
2 bisphosphate (PI(4,5)P₂), its substrate and activator, are excluded (Xie et al.,
3 2002). Chemical or mechanical disruption of lipid raft may release the
4 segregated PLD2 thus enhance the PA production at the plasma membrane
5 (Diz-Munoz et al., 2016; Petersen et al., 2016).

6 PA is a negatively charged, cone-shaped lipid identified as a key mediator in
7 phospholipid metabolism, mTOR activation, membrane trafficking,
8 mitochondrial fusion and actin polymerization (Liu et al., 2013; Yang and
9 Frohman, 2012). PA could be produced through phospholipid synthesis (the
10 Kennedy pathway) in the endoplasmic reticulum, the phosphorylation of
11 diacylglycerol by diacylglycerol kinase, or the hydrolysis of PC by PLD on
12 plasma or endosomal membranes (Liu et al., 2013). Given the activity of PLD2
13 regulated by mechanical cues as well as the importance of its enzymatic
14 product, PA, on actin polymerization and membrane trafficking, we hypothesize
15 that alteration of membrane tension may affect PLD2 activity thus impacts
16 endocytosis. Here, we discover that an acute decrease of plasma membrane
17 tension induces PA production which triggers macropinocytosis without eliciting
18 an increase of PIP₃.

19

20 **Results**

21 **Enrichment of PA at dorsal membrane ruffles upon tension drop**

22 To examine the direct effect of membrane tension on PLD activity, we utilized
23 hypo- or hypertonic buffer to induce the increase or decrease of membrane
24 tension respectively in mouse myoblasts, similar to the approach taken by
25 numerous prior studies (Boulant et al., 2011; Diz-Munoz et al., 2013; Houk et
26 al., 2012). After transfection of a PA biosensor (PABD-GFP, PA binding domain

1 of yeast Spo20p fused with GFP) into C2C12 myoblast, we imaged its
2 distribution with inverted epi-fluorescence microscopy at 37 °C. Similar to a
3 previous report (Zenou-Meyer et al., 2007), PABD-GFP mainly distributed in
4 nucleus and plasma membrane, especially at lamellipodia and membrane
5 ruffles when cells were incubated in an isotonic buffer (1X PBS) (Fig. 1Aa).
6 Interestingly, the enriched signals of PABD-GFP at plasma membrane became
7 diffused after 2 min-incubation in a hypotonic buffer (0.5X PBS) (Fig. 1Ab), and
8 it re-appeared when the cells were recovered by 1X PBS for 2 minutes (Fig.
9 1Ac). We hereafter referred this hypotonic treatment followed with isotonic
10 buffer recovery as osmotic shock (OS) treatment.

11 A similar phenotype could be observed by directly incubating the cells with a
12 hypertonic buffer (PBS + 150 mM sucrose) where PABD-GFP signal at
13 membrane ruffles increased from 2 min, peaked at 5 min and gradually settled
14 (Fig. 1B and S1A). We thus quantified the effect of hypertonic buffer treatment
15 on membrane ruffling by dividing the ruffling phenotype into four categories:
16 sparse, mild, intermediate and severe with <5%, 5-10%, 10-25% or >25% of
17 dorsal area equipped with membrane ruffles respectively (Fig. 1C). While only
18 5% of cells incubated in isotonic buffer had vigorous membrane ruffles
19 (intermediate and severe), 5-min hypertonic buffer incubation significantly
20 increased this population to 52%. Together, we found that PABD-GFP signal at
21 plasma membrane was enhanced when membrane tension was reduced by
22 OS or hypertonic buffer treatment.

23 To test whether the enrichment of PABD-GFP at plasma membrane reflects
24 a change of cellular PA level upon tension alteration, we extracted total lipids
25 from cells treated with different buffers, measured the amount of PA with an
26 enzymatic assay, normalized with protein concentrations and expressed it as

1 fold change compared with control cells. While the total PA amount in
2 hypertonic buffer-treated cells showed similar kinetic accumulation with PABD-
3 GFP signals which increased gradually and peaked at 5 min (Fig. 1D), all the
4 tension manipulations (hypertonic, hypotonic and OS) led to increased total PA
5 (Fig. S1B). These results suggest that total PA quantification may not reflect
6 local PA production or distribution. To specifically investigate the effect of
7 membrane tension on PA dynamics on plasma membrane, we thus mainly used
8 PABD-GFP and live-cell imaging to further dissect this phenomenon.

9 To identify what the PA-rich membrane ruffles induced by tension drop are,
10 we examined several plasma membrane components, including F-actin,
11 dynamin-2 (Dyn2), phosphatidyl-4,5-bisphosphate (PLC-PH-GFP, a probe for
12 PI(4,5)P₂), clathrin-coated pit adaptor protein (μ 2-GFP) and PIP₃ (labeled with
13 Akt-PH-GFP) (Fig. 2A-F). In line with the function of PA on actin polymerization,
14 we observed F-actin enrichment on the PABD-GFP ruffles where the membrane
15 fission enzyme Dyn2 was also localized to (Fig. 2A, B). We thus used Dyn2-
16 mCherry to mark the tension drop-induced membrane ruffles for other
17 membrane components analysis between different pairs of markers.
18 Interestingly, while PI(4,5)P₂ was enriched and co-localized with Dyn2-mCherry,
19 neither PIP₃ nor μ 2-GFP were enriched at those membrane ruffles (Fig. 2C-E).
20 In addition to the enrichment of F-actin on membrane ruffles, dramatic
21 accumulation of cortical actin and actin stress fibers also occurred upon
22 hypertonic buffer treatment (Fig. 2F).

23 To further visualize the membrane ruffles in three dimension (3D) when cell
24 encounters acute tension reduction, we utilized spinning disk confocal
25 microscopy to image the cell before and after hypertonic buffer treatment along
26 the z-axis. After image acquisition and 3D reconstruction, we found that PABD-

1 GFP and Dyn2-mCherry-enriched membrane ruffles were distributed at the
2 peripheral dorsal membrane (open arrow heads in Fig. 2G, H). The decrease
3 of cell volume upon hypertonic buffer incubation could be observed in the
4 orthogonal image where there was a reduction in cell height without changing
5 cell width (Fig. 2I, J). Notably, all the PA and Dyn2-enriched membrane ruffles
6 existed only at the dorsal membrane. Together, we found that an acute
7 decrease of plasma membrane tension results in F-actin and PA-enriched
8 membrane ruffle formation at the peripheral dorsal membrane.

9

10 An acute membrane tension drop induces macropinocytosis

11 The membrane flaccidity-induced F-actin and Dyn2-rich dorsal membrane
12 waves were reminiscent of the membrane ruffles in macropinocytosis. To
13 examine this possibility, we added a macropinocytosis-specific cargo,
14 rhodamine-conjugated dextran (Rh-Dextran) into buffers with different osmotic
15 conditions and incubated PABD-GFP expressing cells with these buffers. While
16 no dextran signal was detected in cells incubated with isotonic buffer, several
17 red puncta with about 1 μm in diameter were observed at the cell periphery in
18 hypertonic and OS-treated cells together with prominent PABD-GFP ruffles (Fig.
19 3A). Quantification results showed that both OS and hypertonic buffer
20 treatments result in ~ 20% cells obtaining ≥ 3 dextran-positive macropinosomes
21 (Fig. 3B). This result suggests that acute tension reduction induced by either
22 direct hypertonic buffer incubation or OS treatment results in PA production,
23 actin and Dyn2-rich membrane ruffling and subsequently macropinocytosis.

24

25 PLD2 is responsible for PA production upon tension decrease

26 To examine whether PLD2 is responsible for the tension induced-PA

1 production, we first used a general PLD inhibitor 1-butanol (Brown et al., 2007),
 2 to pre-treat myoblasts for 10 min and then incubated the cells with hypertonic
 3 buffer (Fig. S2). After 10 min incubation with 0.1% 1-butanol in isotonic buffer,
 4 mild cell contraction and diminished PABD-GFP signal on plasma membrane
 5 were observed (Fig. S2A). Further contraction of the cell was observed after
 6 hypertonic buffer treatment, yet no membrane ruffles were induced. By contrast,
 7 the control alcohol 2-butanol-treated cells showed prominent membrane ruffling
 8 enriched with PABD-GFP and Dyn2-mCherry upon tension drop. (Fig. S2B).
 9 This result indicates that PLD activity is essential for the PA-rich membrane
 10 ruffles induced by tension drop.

11 Mammalian cells express mainly two isoforms of PLD, PLD1 and PLD2,
 12 which are localized to intracellular membrane and plasma membrane,
 13 respectively. To examine the involvement of these two PLDs, we first monitored
 14 the distribution of PLD1 and PLD2 in cells treated with hypertonic buffer. While
 15 GFP-PLD1 remained intracellularly distributed in hypertonic buffer, GFP-PLD2
 16 showed increased signals at dorsal membrane ruffles upon tension reduction
 17 (Fig. 3C). To further test the specific requirement of PLD2 activity upon tension
 18 drop, we used PLD1 or PLD2 specific inhibitors, UV0359595 and UV-364739
 19 respectively, to pre-treat the cells for 10 min in 1X PBS and followed by 5-min
 20 hypertonic buffer incubation in the presence of inhibitor. Consistent with the
 21 subcellular localization results, we found that only PLD2 inhibitor blocked the
 22 membrane ruffling induced by tension drop, but not in PLD1 inhibitor-treated
 23 cells (Fig. 3D). Consistent with the data above, only the PLD2 inhibitor blocked
 24 Rh-Dextran internalization induced by hypertonic buffer incubation (Fig. 3E, F).

25 To substantiate the specific requirement of PLD2 in tension drop-induced
 26 macropinocytosis, we knocked down (KD) either PLD1 or PLD2 with two

1 different lentiviral shRNA sequences. Similar with the results of PLD inhibitors,
2 we observed significant reduction of membrane ruffles and macropinosome
3 formation in PLD2 KD cells, not PLD1 KD cells (Fig. 4A-C). It is worth noting
4 that when PLD1 or PLD2 were knocked down, the expression of the other
5 isoform was slightly increased (Fig. 4D), indicating a compensatory expression
6 and adaptation phenomenon of cells with PLD depletion.

7 Together, these results suggest that membrane flaccidity induces PA
8 production and macropinocytosis *via* PLD2 activity. Notably, the intracellular
9 GFP-PLD1 vesicles were diminished upon tension surge concurrent with the
10 increased GFP-PLD1 signals on plasma membrane (Fig. S3A), whereas GFP-
11 PLD2 was less enriched on plasma membrane with hypotonic buffer treatment
12 (Fig. S3B). These results indicate that both PLDs respond to tension change
13 and may explain why both an increase and a decrease of membrane tension
14 caused the increase of total PA amount (Fig. S1B).

15

16 **Lipid microdomain responds to tension alteration**

17 Lipid raft has been reported as a mechanosensor and could be disrupted
18 chemically or mechanically (Oglecka et al., 2014; Petersen et al., 2016). We
19 wondered whether the integrity of lipid microdomain may be affected by
20 membrane tension *in vitro* and *in vivo*. To directly observe the effect of
21 membrane tension on lipid microdomain, we utilized giant unilamellar vesicles
22 (GUVs), a commonly used membrane template for studying lipid phase
23 separation, to monitor the size of different lipid phases with L_o (liquid-ordered)
24 domain labeled with Topfluor cholesterol and L_d (liquid-disordered) domain
25 labeled with rhodamine-PE. In GUVs composed of
26 DOPC:DPPC:Cholesterol:Topfluor cholesterol: rhodamine-PE with molar ratio

1 at 39:39:20:1:1, we observed phase separation with several L_o domains
 2 (around 3 – 15 per GUV) when the GUVs were incubated in isotonic buffer (Fig.
 3 5A and video 1). Strikingly, when GUVs were incubated in hypotonic buffer,
 4 those L_o domains merged into one large domain with numerous small L_o
 5 domains continuously formed and fused together (Fig. 5A and video 2). By
 6 contrast, L_o domains became smaller in hypertonic buffer where GUVs on a
 7 coverslip resemble flatten tires (Fig. 5A and video 3). These results convincingly
 8 showed that membrane tension affects the integrity of lipid microdomain on
 9 model membrane.

10 To test whether the flaccidity-induced disruption of lipid microdomain could
 11 be observed in a cell, we utilized Alexa fluor 647-conjugated cholera toxin B
 12 (CTB) which binds to sphingolipid GM1 to label lipid raft and monitored its
 13 distribution together with GFP-PLD2. Upon different osmotic buffer treatments,
 14 fixation and CTB-AF 647 staining, images of dorsal membranes were acquired
 15 with Stochastic Optical Reconstruction Microscopy (STORM) and analyzed with
 16 ThunderSTORM plugin in ImageJ (Fig. 5B). We observed partial co-localization
 17 between GFP-PLD2 and CTB labeled lipid microdomains in hypertonic buffer-
 18 treated cells; however, the size of CTB-labeled microdomains were similar
 19 among the three conditions. We further used Stimulated Emission Depletion
 20 (STED) Microscopy to image the ventral surface of myoblasts after indicated
 21 osmotic buffer treatments and found no significance difference in the size of
 22 CTB-AF 594 puncta in different buffer-treated cells (Fig. 5Ca-c), though the
 23 CTB clusters seemed less compact in hypertonic buffer-treated cells (arrow
 24 heads in Fig. 5D).

25 To quantitatively examine the effect of tension change in cells, we utilized
 26 nonionic detergent to isolate detergent-resistant membrane under different

1 tension conditions (Lingwood and Simons, 2007). Given the weak signals of
 2 endogenous PLD2 in C2C12 myoblast (Fig. 4D), we ectopically expressed
 3 GFP-PLD2 in myoblast, treated the cells with buffers with distinct osmolarity
 4 and isolated the detergent-resistant membrane with 1% cold TX-100 and
 5 sucrose gradient ultracentrifugation, then detected the distribution of interested
 6 proteins with Western blotting (Fig. 5E-H). In addition to GFP-PLD2, we also
 7 detected other membrane proteins, including PLD1, caveolin-1 and Na⁺-K⁺
 8 ATPase for comparison. Both GFP-PLD2 and caveolin-1 became less resistant
 9 to detergent (less enrichment in fractions 2-4) in cells at a lower tension (Fig.
 10 5F), supporting the idea that tension drop destabilized lipid rafts. In contrast,
 11 lipid rafts remained intact in cells with increased membrane tension (Fig. 5G,H).
 12 Interestingly, PLD1 partially shifted to detergent-resistant membrane fractions
 13 in hypotonic condition that is consistent with its partial redistribution to plasma
 14 membrane upon tension surge (Fig. 5G and S3A). Together, these results
 15 demonstrate that membrane tension facilitates lipid phase separation, thus a
 16 decrease of tension results in lipid raft destabilization *in vitro* and *in vivo*.

17

18 **Acute decrease in membrane tension in myotube induces** 19 **macropinosome formation**

20 Given the results above, we have identified a previously underappreciated
 21 pathway which transduces mechanical cue into biochemical signal, *i.e.* the
 22 membrane tension alteration leads to phosphatidic acid production. However,
 23 compared to the pronounced actin polymerization (Fig. 2F), we reasoned the
 24 relatively small amount of membrane ruffles and macropinosome in C2C12
 25 myoblast upon tension drop indicates that PA-induced macropinocytosis
 26 contributes to a limited extent for cell to respond to tension alteration. We thus

1 wonder whether this pathway will be more pronounced in cells with restricted
2 actin dynamics, for example, in the muscle cells.

3 Skeletal muscle is constantly exposed to mechanical stress in our body thus
4 has been intensively studied for its ability to cope with increased membrane
5 tension. Muscle cell is equipped with robust caveolae structures which could
6 quickly disassemble when membrane tension increases in order to release
7 membrane reservoir thus relieve the tension surge (Lo et al., 2015; Sinha et al.,
8 2011). However, little is known about how muscle cells react upon rapid
9 membrane tension drop following a tension surge. After hypertonic buffer or OS
10 treatment in the presence of Rh-Dextran, we observed significant amounts of
11 dextran-containing macropinosomes in C2C12-derived myotubes (Fig. 6A).
12 Remarkably, the macropinosomes induced by OS were much larger than the
13 ones in hypertonic buffer-treated myotubes which could be observed under light
14 microscopy (Fig. 6Bc, red arrow heads). This is probably due to the increase of
15 membrane area by caveolae disassembly during hypotonic treatment (black
16 arrow in Fig. 6Bb) thus results in more pronounced macropinosomes than cells
17 in hypertonic buffer incubation. Furthermore, using time-lapse microscopy, we
18 found those OS-induced, dextran-positive macropinosomes were also enriched
19 with PA (Fig. 6C).

20 To examine whether macropinocytosis induced by OS treatment is
21 physiologically relevant, we stretched and relaxed PABD-GFP expressing
22 myotubes with radial cell stretcher for 2 min-stretching and 2 min-relaxation in
23 the presence of Rh-Dextran in medium. After wash and fixation, cells were
24 imaged with confocal microscopy and we observed some macropinosomes
25 labeled with dextran and PABD-GFP in myotubes, but not in the control
26 myotubes which were incubated in dextran without stretching (Fig. 6D). These

1 results demonstrate that acute membrane tension reduction induced by
2 intensive cell stretching and relaxation, similar to OS treatment, triggers PA
3 production and macropinocytosis. We hereafter utilized OS-treated myotubes
4 to observe the kinetic and PLD contribution to tension change-induced
5 macropinocytosis in myotube.

6 In contrast to what we observed in myoblast, there was no PA-rich membrane
7 ruffles in myotubes upon OS treatment (Fig. 6B,C). Thus, we carefully
8 examined the kinetic distribution of Dyn2-mCherry and PABD-GFP in myotube
9 treated with OS (Fig. 7A). Although there was no membrane ruffling detected in
10 OS-treated myotubes, we found Dyn2-mCherry enriched at PABD-GFP ring at
11 membrane during the early stage of OS treatment (Fig. 7A, inset). Interestingly,
12 only when the macropinosome was formed above the nucleus where much less
13 cytosolic signals exist that the Dyn2-mCherry could be observed on the
14 membrane, potentially the intermediate stage of macropinocytosis (Fig. 7A). In
15 addition, different from the PA-labeled macropinosomes that could last for more
16 than 10 min in myotubes, the Dyn2-mCherry ring quickly disappeared from
17 plasma membrane upon OS treatment, suggesting the disappearance of Dyn2
18 or the disassembly of Dyn2 ring after membrane scission (Antonny et al., 2016).
19 Together, these results suggest that an acute membrane tension decrease also
20 induced PA and Dyn2-mediated macropinocytosis in myotube though without
21 displaying a membrane ruffling phenotype.

22 Next, we asked whether PLD is also responsible for the tension change-
23 induced macropinocytosis in myotube. Interestingly, PLD1-GFP distributed both
24 on plasma membrane and intracellular vesicles in myotube and partially
25 distributed to macropinosomes after OS, whereas PLD2 was localized at the
26 plasma membrane in isotonic condition and became enriched at

1 macropinosomes upon OS treatment (Fig. 7B,C). Consistent with these
2 observations, OS-induced macropinosome formation in myotubes could only
3 be blocked by the combined treatment of PLD1 and PLD2 inhibitors (Fig. 7D).
4 Together, these data indicate that the tension drop-PLD-macropinocytosis
5 pathway is dominant in myotubes and both PLD1 and PLD2 are involved.

6

7 **PA enhances the membrane fission activity of Dyn2**

8 PA is known to promote actin polymerization through PI(4,5)P₂ production
9 and Rac activation (Liu et al., 2013). However, it has not been addressed
10 whether PA could directly affect the activity of Dyn2 which is a membrane fission
11 GTPase essential for phagocytosis and macropinocytosis. (Liu et al., 2008;
12 Marie-Anais et al., 2016). Furthermore, the fission activity of Dyn2 is tightly
13 regulated by membrane curvature and lipid composition (Morlot et al., 2012;
14 Pucadyil and Schmid, 2008; Roux et al., 2010). To test whether PA could
15 facilitate the activity of Dyn2, we first performed liposome sedimentation assays
16 to examine the membrane binding ability of purified human Dyn2 to liposomes
17 containing different amount of PA. There was no significant difference of Dyn2
18 binding to PA-containing liposomes (Fig. S4A, B). Interestingly, the GTPase
19 activity of Dyn2 increased in a PA-dependent manner (Fig. S4C). We further
20 analyzed the assembly of Dyn2 on liposomes and its membrane fission activity
21 with negative stain electron microscopy and *in vitro* membrane fission assay.
22 We found that not only did Dyn2 assemble into much ordered and distinct
23 helical structures in PA-containing liposomes (Fig. S4D), it also showed higher
24 fission activity on PA-containing membrane template (Fig. S4E). Interestingly,
25 the fission activity of Dyn2 seemed lower on 10% PA membrane compared to
26 5% PA membrane which is probably due to the instability and spontaneous

1 membrane breakage on membrane reservoir made with high amount of PA.
2 Together, PA facilitates the proper assembly of Dyn2 on membrane without
3 changing the fraction bound, thus enhances its GTP hydrolysis and membrane
4 fission activities.

5

6 **Discussion**

7 Macropinocytosis is a unique endocytic pathway which is initiated by
8 biochemical stimulations to efficiently uptake nutrients, terminate signaling or
9 internalize viruses (Buckley and King, 2017). In this study, we identified a PLD-
10 dependent, but PIP₃-independent pathway of macropinocytosis that is induced
11 by acute membrane tension drop which leads to lipid raft destabilization, PLD2
12 activity, PA production and macropinosome formation (Fig. 8). This particular
13 mechanotransduction pathway contributes differentially among cell types and
14 is much pronounced in myotubes and reflects the molecular mechanism of
15 muscle cell coping with intensive stretching and relaxation during physical
16 exercise.

17 Lipid raft is well perceived as a signaling platform given its enrichment of
18 unique receptors. Here we find it also serves as a mechanosensor which
19 undergoes disruption upon membrane tension alteration to activate PLD2. The
20 effect of membrane tension of the coarsening of lipid microdomains *in vitro* has
21 been largely studied and modeled theoretically (Akimov et al., 2007; Chen and
22 Santore, 2014; Hamada et al., 2011; Oglecka et al., 2014; Portet et al., 2012;
23 Ursell et al., 2009). It is believed that the membrane tension sensitivity of lipid
24 microdomain may arise from (1) the increase of line tension when membrane
25 tension rises (Akimov et al., 2007), or (2) the kinetics of microdomain
26 coalescence affected by membrane tension (Ursell et al., 2009). Line tension is

1 the boundary energy between L_o and L_d domains that comes from the difference
 2 of their membrane thickness, also known as hydrophobic mismatch. Therefore,
 3 it is intuitive to imagine that membrane with lower tension would have lower
 4 boundary energy thus the total circumference of L_o could increase, *i.e.* a larger
 5 number of L_o domains with smaller diameters (Fig. 8). With that, it is tempting
 6 to hypothesize that PLD2 catalyzes reactions at the boundary of lipid rafts and
 7 the effect of membrane tension on lipid raft is to increase the amount of phase
 8 boundaries which leads to higher PLD2 activity.

9 The activity of PLDs has been reported to be sensitive to mechanical stress
 10 such as mechanical stretching, membrane tension surge or osmotic stress (Diz-
 11 Munoz et al., 2016; Hornberger et al., 2006; Tomassen et al., 2004). However,
 12 how exactly PLD is activated upon mechanical stimulation remains unclear.
 13 Here, we found that PLD1 and PLD2 distribute differently and react to distinct
 14 tension alterations in myoblasts. Nonetheless, it is puzzling that both an
 15 increase and a decrease of membrane tension result in elevated total amount
 16 of PA, yet resulted in different cellular outcomes: actin depolymerization or
 17 polymerization, respectively (Diz-Munoz et al., 2016; Tsujita et al., 2015). Since
 18 the subcellular location of PA and its surrounding proteins determine where and
 19 what kind of cellular event it would participate (Colley et al., 1997; Du et al.,
 20 2004; Teng et al., 2015; Yang and Frohman, 2012), it is plausible that elevated
 21 PA in different membrane compartment would trigger distinct cellular processes.
 22 Furthermore, given that high membrane tension directly inhibits actin
 23 polymerization and the force feedback mechanism on actin assembly (Bieling
 24 et al., 2016; Houk et al., 2012; Tsujita et al., 2015), the effects of membrane
 25 tension on PA production are important contributors to the final phenotype
 26 outputs. Further studies are needed to elucidate the feedback regulation of

1 membrane tension, local PA production and actin polymerization in order to fully
2 deciphering the complexity of this network.

3 PA has a small phosphate head group thus could create lipid packing defect
4 in membrane by its nature (Bigay and Antonny, 2012). Here we found that
5 membrane with PA facilitates the assembly of Dyn2, but not its binding, which
6 is reminiscent of the effect of membrane curvature on Dyn2 assembly and
7 activity (Liu et al., 2011a; Roux et al., 2010). Curved membrane provides more
8 lipid packing defect thus facilitates the insertion of the PH domain in Dyn2 to
9 have better assembly. With that, membrane with more PA may have more lipid
10 packing defects due to its geometry. Consistent with this notion, PA has been
11 reported to induce the insertion of Dyn1 into lipid monolayer (Burger et al.,
12 2000). Together, these results suggest that Dyn2 assembly is better positioned
13 for its fission activity on PA-containing membrane without the prerequisite of
14 membrane curvature.

15 The mechanotransduction pathway we discovered here supports the concept
16 that membrane tension is an integrator for biochemical and physical cues to
17 regulate membrane trafficking and actin organization. By examining the
18 molecular basis of this regulatory network, we uncovered the mechanisms
19 underpinning membrane organization and PLD activity. Given the ubiquitous
20 nature of forces, we suspect there are more molecules that sense and/or are
21 regulated by membrane tension. Our findings pave the way for future studies
22 of the mechanical regulation on membrane and cell physiology.

23

24 **Materials and Methods**

25 **Cell Culture, Transfection and Infection**

26 Mouse-derived C2C12 myoblasts (American Type Culture Collection, CRL-

1 1772) were cultured in growth medium (GM), DMEM supplemented with 2 mM
2 L-glutamine, 1 mM sodium pyruvate, antibiotics and 10% fetal bovine serum
3 (Gibco). To induce differentiation, C2C12 were seeded onto laminin
4 (Invitrogen)-coated glass-bottom dish (MatTek) or coverslips in GM, grown to
5 90% confluency, and then switched to differentiation medium (DM), which is the
6 same as GM but with 2% horse serum (Gibco). This time point was considered
7 as day 0 of differentiation. For transfection, cells at 70% confluency were
8 transfected with the desired DNA constructs using Lipofectamine 2000
9 (Invitrogen), as recommended by the manufacturer.

10 For PLD knockdown experiments, lentiviruses with shRNA sequences:
11 5'-CCCAATGATGAAGTACACAAT-3' or 5'-GCTTGGTAATAAGTGGATAAA-3'
12 for PLD1; whereas 5'-CCTTCCTGTCACCAAGTTCAA-3' or 5'-
13 CATGTCTTTCTATCGCAATTA-3' for PLD2, were prepared. C2C12 myoblast
14 were infected with control or PLD-shRNA containing lentiviruses and selected
15 with 2 µg/ml puromycin for 3 days then subjected to transfection and osmotic
16 buffer treatments.

17

18 **Reagents**

19 PLD1 inhibitor UV0359595 was purchased from Cayman Chemical. PLD2
20 inhibitor UV0364739 was from Tocris Bioscience. Rh-dextran, CTB-AF 647 and
21 CTB-AF 594 were from Invitrogen. Anti-PLD2 antibody was purchased from
22 Abcam, anti-PLD1 and anti-caveolin-1 antibodies were from Cell Signaling
23 Technology, anti-Na,K-ATPase antibody was from Santa Cruz Biotechnology,
24 and anti-GFP antibody was a gift from Fang-Jen S. Lee in National Taiwan
25 University. All lipids were purchased from Avanti Polar Lipids.

26

1 **Microscopy**

2 For live-cell microscopy, cells transfected with interested DNA constructs were
 3 seeded on glass-bottom dish (MatTek) and imaged with Zeiss inverted
 4 microscopy Axio Observer Z1 or spinning disc confocal microscope (Carl Zeiss
 5 Observer SD) at 37 °C with 63x, 1.35-NA oil-immersion objective. To image
 6 fixed cells, sample slides were observed with confocal microscopy LSM700 with
 7 63x, 1.35-NA oil-immersion objective (Carl Zeiss, Jena, Germany).

8 For super-resolution microscopy, we used both STORM and STED to
 9 image cells upon hyper- or hypo-osmotic shock. For STORM, GFP-PLD2
 10 transfected C2C12 myoblasts were subjected to iso- (1X PBS), hypo- (0.5X
 11 PBS), or hypertonic (1X PBS with 150 mM sucrose) buffers for 5 minutes. After
 12 4% paraformaldehyde fixation and intensive PBS wash, cells were incubated
 13 with Alexa fluor 647-cholera toxin B (CTB, 1 µg/ml, Invitrogen) overnight at 4
 14 °C. After PBS wash, the STORM buffer (50 µL beta-mercaptoethanol (BME),
 15 1.85 mM monoethanolamine (MEA), 0.25 M NaCl, 50 mM Tris with a final pH
 16 of 9.0) was added with protocatechuic acid (PCA)/protocatechuate-3,4-
 17 dioxygenase (PCD) O₂-scavenging system immediately before imaging was
 18 used for switching the fluorophores. The imaging was performed at the Single
 19 Molecule Analysis in Real-Time (SMART) Center, University of Michigan, using
 20 computer-controlled IOX-81 Olympus microscope with 60x oil 1.49NA
 21 (APON60XOTIRFM) and an Andor iXon Ultra EM-CCD camera. 10000 images
 22 of Af 647-CTB excited with 640 nm (Coherent Cube) laser were collected at
 23 100 ms exposure time using stream acquisition. After acquisition of 5000
 24 images with 640 nm laser illumination, sample was co-exposed to 405 nm
 25 (Coherent Cube) laser to excite fluorophores to triplet state. Samples were
 26 exposed to both 640 nm and 405 nm until acquisition of 10000 images. A GFP-

1 PLD2 image was taken before and after the STORM imaging. The STORM
2 image reconstruction was performed using ThunderSTORM plugin in ImageJ,
3 using difference of Gaussian image filtering and sub-pixel localization of
4 molecules using maximum likelihood fitting with integrated Gaussian PSF.

5 For STED imaging, cells treated with different osmotic buffers and
6 followed by fixation and Alexa fluor 594-CTB (Invitrogen) staining were
7 mounted in ProLong Gold antifade reagent (Invitrogen) and imaged under Leica
8 TSC SP8 X STED 3X with 100x oil objective 1.4NA (STED). Samples were
9 acquired with excitation laser 594 nm, 660 nm depletion laser and Hybrid
10 Detector (Leica HyD).

11

12 **PA measurement**

13 Total PA content was measured with a coupled enzymatic reaction assay (Total
14 Phosphatidic Acid Assay Kit, Cell Biolabs, Inc.). Briefly, cells treated with buffers
15 of different osmolarities were scraped from dishes. While 10% of the samples
16 were used for protein concentration analysis, the rest of the samples were
17 subjected to total lipid extraction by methanol and chloroform. After drying with
18 SpeedVac, the lipid film was dissolved and the amount of PA was determined
19 by a fluorometric assay that firstly hydrolyzed PA to glycerol-3-phosphate by
20 lipase. Next, glycerol-3-phosphate product was oxidized by glycerol-3-
21 phosphate oxidase, producing hydrogen peroxide which reacted with a
22 fluorometric probe with excitation/emission wavelength at 530-560 nm/585-
23 595nm. The PA amount was determined as fluorescence intensity (A.U.)/protein
24 concentration (μg).

25

26 **Macropinocytosis assay**

1 To monitor macropinocytosis, myoblasts or myotubes were incubated with
 2 indicated osmotic buffers containing 1 μ g/ml Tetramethylrhodamine Dextran
 3 (Thermo Fisher Scientific). After PBS wash for five times, cells were fixed and
 4 imaged with confocal microscopy. To monitor macropinocytosis in myotube
 5 upon cell stretching, myoblasts were seeded on laminin-coated flexcell silicon
 6 dish and were induced to differentiate for five days after PABD-GFP transfection.
 7 Day-5 differentiated myotubes were incubated in DM medium containing 1
 8 μ g/ml Rh-Dextran and subjected to radial stretching of 2 min at 20% extension
 9 and followed by 2 min resting. After intensive wash, fixation and mounting, cells
 10 were imaged by confocal microscopy.

11

12 **Fractionations of detergent-resistant membrane domains**

13 Detergent-resistant membrane domains were purified from cells exposed to
 14 buffers with different osmolarities as previously described (Czarny et al., 1999).
 15 Briefly, after incubation with buffers of different osmolarity, myoblasts (two sub-
 16 confluent 100-mm dishes) were scraped into ice-cold buffers with the same
 17 osmolarity as previous treatment. After centrifugation, the pelleted cells were
 18 re-suspended with 0.8 ml lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM
 19 EDTA, 1% Triton X-100 and proteinase inhibitor cocktail (Roche)). After 30 min
 20 incubation at 4 °C, cell extracts were adjusted to 40% sucrose by addition of
 21 0.8 ml of the above buffer (minus Triton X-100) containing 80% sucrose and is
 22 then placed at the bottom of a 5-ml ultracentrifuge tube. A step sucrose gradient
 23 was formed above the lysate by adding 2.4 ml of 35% and 0.8 ml of 5% sucrose
 24 solutions, and the tubes were centrifuged at 150,000 \times g for 8 h in a SW-55 Ti
 25 rotor at 4 °C. Fractions (0.4 ml) were collected from the top of the gradient and
 26 analyzed with Western blotting.

1

2 **Liposome and GUV preparation**

3 For liposomes preparation, lipid mixtures (DOPC:DOPS:PI(4,5)P₂:DOPA at 80-
4 70:15:5:0-10) were dried, rehydrated in HK buffer (20 mM HEPES (pH 7.5), 150
5 mM KCl) and subjected to a series of freeze–thaw cycles before extrusion
6 through polycarbonate membranes (Whatman) with pore sizes of 100 or 400
7 nm using an Avanti Mini-Extruder. For GUV formation, lipid mixtures
8 (DOPC:DPPC:cholesterol:Rhodamine-PE:Topfluor-cholesterol at 39:39:20:1:1)
9 were dried on a ITO-coated glass slide and electroformation was conducted *via*
10 applying 3.5V for 3 hour in 400 mM sucrose at 50 °C. To monitor the effect of
11 tension changes on lipid phase separation, freshly prepared GUVs were
12 subjected to isotonic buffer (400 mM glucose), hypertonic buffer (500 mM
13 glucose) or hypotonic buffer (300 mM glucose) and imaged with inverted
14 fluorescence microscopy immediately.

15

16 **Myotube stretching and imaging**

17 C2C12 cells seeded on laminin-coated BioFlex Culture Plate (Flexcell) was
18 transfected with PABD-GFP and differentiated into myotube by DM incubation.
19 After five days of differentiation, Rhodamine-Dextran was added into the
20 medium to reach 1 µg/ml, and myotube was subjected to static, radial stretching
21 with 20% strain by a Flexcell FX-5000T Tension System for 2 min and followed
22 by no stretching for 2 mins; whereas control cells were incubated in Rh-Dextran
23 containing medium for 4 mins without mechanical stress. Samples were then
24 washed with PBS for 5 times, fixed with 4% formaldehyde for 30 min, cut from
25 the plates and mounted with mounting medium and coverslip. PABD-GFP and
26 Rh-Dextran signals were imaged with confocal microscopy (Zeiss, LSM700).

1

2 **Dynamin purification and activity analysis**

3 Recombinant human Dyn2 was purified from insect cells and stored at -80 °C
 4 as previously described (Liu et al., 2011b). To determine membrane binding,
 5 0.5 μM Dyn2 were incubated with 150 μM, 400 nm-sized liposomes with
 6 different amount of PA at 37 °C for 15 min. After centrifugation at 20,000 xg for
 7 30 min, liposome-bound Dyn2 was pelleted down and was quantified by SDS-
 8 PAGE analysis. To analyze GTPase activity, GTP hydrolysis by Dyn2 was
 9 measured as a function of time using a colorimetric malachite green assay to
 10 detect the release of inorganic phosphate (Leonard et al., 2005). Briefly, 0.5 μM
 11 Dyn2 was incubated with 150 μM liposomes of different PA concentrations in a
 12 buffer containing 20 mM HEPES, pH 7.4, 150 mM KC, 1 mM GTP and 2 mM
 13 MgCl₂. Aliquots were taken at several time points, free phosphate was
 14 determined using malachite green, and rates of hydrolysis were calculated.
 15 Fission activity of dynamin was measured as previously reported (Liu et al.,
 16 2011b). Briefly, the supported bilayers with excess membrane reservoir
 17 (SUPER) templates with different amount PA were incubated with 1 mM MgCl₂,
 18 1 mM GTP, and indicated dynamin for 30 min at room temperature. The
 19 released vesicles were separated from SUPER template by 260 xg swing-out
 20 centrifugation. The fission activity is expressed as percentage of total
 21 fluorescence on SUPER templates.

22

23 **Transmission electron microscopy**

24 To visualize the assembly of Dyn2 on liposomes, 1.0 μM dynamin was mixed
 25 with 25 μM liposomes with different lipid compositions and incubated at 37 °C
 26 for 10 min. The mixture was then adsorbed onto carbon-coated, glow

1 discharged grids and stained with 1% uranyl acetate. Images were collected
2 using a Hitachi H-7650 EM at 75 kV and a nominal magnification of $\times 120,000$.

3

4 **Statistical analysis**

5 Quantitative data were expressed as mean \pm SD of at least three independent
6 experiments. All data were analyzed with One-way ANOVA, except the DRM
7 isolation result was analyzed with Student's *t*-test. $p < 0.05$ was considered as
8 statistical significance p values were indicated as *, $p < 0.05$; **, $p < 0.01$; ***,
9 $p < 0.001$.

10

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21

22 **Author contributions**

23 All authors participated in experimental design. JL, JJ, MCC, SSL, YCC, YAS
24 and YWL performed experiments. JL, JJ, APL and YWL analyzed data and
25 wrote the manuscript. APL and YWL supervised the project.

26

1 **Declarations of interests**

2 The authors declare no competing financial interests.

3

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Figure Legends

Figure 1. Effects of membrane tension manipulation on PA in myoblast.

(A) An increase of membrane tension suppresses PA-rich ruffles at plasma membrane. PABD-GFP expressing C2C12 myoblast was first imaged in isotonic buffer (Aa, 1X PBS), and two images were further captured after 2 min of hypotonic buffer (Ab, 0.5XPBS) and additional 2 min of 1X PBS recovery (Ac). Boxed areas were magnified and shown on the right panel. (B) A decrease of tension induces PA enrichment at plasma membrane. PABD-GFP expressing myoblast was first imaged in isotonic buffer (Ba), and two images were further captured after 2 min (Bb) or 5 min (Bc) after hypertonic buffer treatment (1XPBS + 150 mM sucrose). (C) Quantification of membrane ruffling in cells treated with isotonic or 5-min hypertonic buffers. Ruffling phenotypes were divided into four categories with different levels or areas of membrane wave. (D) Kinetic change of total PA amount in myoblasts upon hypertonic buffer treatment. C2C12 myoblasts treated with hypertonic buffer for indicated time period were harvested and analyzed with total PA assay and normalized with the protein concentration. Fold change of the PA/protein ratio was compared with isotonic buffer treatment. Scale bars, 10 μ m. *, $p < 0.05$.

Figure 2. Distribution of membrane proteins and lipids upon sudden decrease of membrane tension.

(A-F) Myoblasts co-transfected with indicated plasmids were treated with hypertonic buffer for 5 mins. The tension drop-induced PA-rich ruffles were examined for its enrichment with F-actin (Lifeact-RFP), Dyn2, clathrin-coated pit adaptor (μ 2-GFP), PI(4,5) P_2 (PLC-PH-GFP) and PI(3,4,5) P_3 (Akt-PH-GFP). (G-J) Spinning disc confocal microscopy images of myoblast incubated in a hypertonic buffer. PABD-GFP and Dyn2-mCherry co-transfected myoblast was imaged with z-stack confocal microscopy before and after 5 min incubation in a hypertonic buffer. The 3D reconstructed images (G,H) and orthogonal views (I,J) were shown to illustrate dorsal membrane ruffling (open arrow heads in H and white arrow heads in J) upon tension drop. Scale bars, 10 μ m.

Figure 3. Acute membrane tension drop induces PLD2-dependent

macropinocytosis. (A) Macropinocytosis is induced upon hypertonic buffer or OS treatment. PABD-GFP expressing myoblasts were incubated with indicated buffer plus 1 $\mu\text{g/ml}$ Rh-Dextran, including 5 min isotonic (1X PBS), 5 min hypertonic (1XPBS + 150 mM sucrose) or 2 min hypotonic (0.5X PBS without dextran) followed with isotonic (1X PBS with dextran) for OS. After wash and fixation, cells were imaged with confocal microscopy and the maximum intensity projection images were generated. (B) The number of macropinosomes (Rh-Dextran containing vesicle larger than 0.5 μm in diameter) were quantified and divided into three populations as indicated. Percentage of each population were compared with the isotonic buffer-treated cells, and over 160 cells for each condition were analyzed. (C) PLD1 and PLD2 distribution upon membrane tension drop. Myoblasts expressing GFP-PLD1 or GFP-PLD2 were imaged before (Ca and Cc) and after (Cb and Cd) hypertonic buffer treatment. (D-F) Effect of PLD inhibitors on tension drop-induced membrane ruffles and macropinocytosis. PABD-GFP expressing myoblasts were pretreated with indicated PLD inhibitors, 500 nM for 10 min in 1X PBS, and followed by inhibitor-containing hypertonic buffer for another 5 min (D) or Rh-Dextran containing hypertonic buffer and OS treatment (E,F). Live cell imaging (D) or confocal imaging on fixed cells (E) were performed respectively. (F) The population of cells with different numbers of macropinosome were quantified with 150 cells of each condition and compared with vehicle control. Scale bars, 10 μm . *, $p < 0.05$; **, $p < 0.01$.

Figure 4. PLD2 is required for tension drop-induced membrane ruffling and macropinocytosis. (A) PABD-GFP transfected control, PLD1- or PLD2-depleted myoblasts were subjected to hypertonic buffer treatment, and the distribution of PABD-GFP on dorsal membrane was captured by inverted fluorescence microscopy. (B) Macropinocytosis of PABD-GFP myoblasts upon hypertonic buffer treatment were monitored with Rh-Dextran internalization in cells PLD1 and PLD2 depleted cells compared to control. (C) The population of cells with more than three macropinosomes (the diameter $\geq 0.5 \mu\text{m}$) as determined by Rh-Dextran for experimental conditions in (B) were quantified and compared with control cells. 120 cells of each condition were scored. (D) Western blotting of PLD1 and PLD2 in myoblast upon PLD shRNA KD. Scale bars, 10 μm . **, $p < 0.01$.

Figure 5. Lipid microdomain disruption upon tension drop. (A) Effects of tension changes on lipid phase separation. GUVs with Topflour cholesterol

(green) and rhodamine-PE (magenta) to label L_o and L_d domains respectively were subjected to iso- (400 mM), hyper- (500 mM), or hypo-tonic (300 mM) glucose buffers and imaged with inverted epi-fluorescence microscopy. Scale bar, 10 μ m. (B,C) Effects of hyper- or hypotonic buffer treatment on lipid rafts in cell. Myoblasts were treated with indicated buffers, fixed, stained with CTB and imaged with epi-fluorescence (GFP-PLD2) or STORM (CTB-AF 647) (B) or STED microscopy (C), respectively. (D) Boxed areas in (C) were magnified. (E-G) Fractionations of detergent-resistant membrane proteins upon tension changes. GFP-PLD2 expressing myoblasts treated with indicated osmotic buffers were harvested and lysed with buffer containing 1% Triton X-100 at 4 °C for 30 min and subjected to sucrose gradient ultracentrifugation. Detergent resistant membranes were distributed in fraction 2-4. (H) The ratio of GFP-PLD2 in each fraction was analyzed with Student *t*-test. Three repeats of the experiments were quantified and compared with isotonic buffer-treated cells. *, $p < 0.05$.

Figure 6. Acute tension drop induces macropinocytosis in myotubes. (A) Macropinosome formation in myotubes. C2C12-derived myotubes incubated in indicated buffers plus Rh-Dextran. After intensive wash, cells were fixed, stained with DAPI and imaged with confocal microscopy. (B) Morphology of myotubes treated with isotonic buffer (Ba), hypotonic buffer (Bb) and followed by isotonic buffer recovery (Bc). Phase contrast microscopy images were captured with inverted fluorescence microscopy. Black arrow indicates the membrane bleb from cell swelling, and the red arrow heads point to macropinosomes induced by tension drop. (C) Macropinosomes are enriched with PA in OS-treated myotube. Live cell imaging of PABD-GFP expressing myotube was captured when cells were incubated in isotonic buffer (Ca), hypotonic buffer (Cb) and recovery isotonic buffer containing 1 μ g/ml Rh-Dextran for 2 min with intensive wash (Cc). Insets are magnified images from the boxed areas. (D) Radial stretch induces macropinosome formation in myotubes. PABD-GFP expressing myotubes incubated in 1 μ g/ml Rh-Dextran medium were subjected to 0% or 20% radial stretch for 2 min and followed by 2 min resting (0% stretch). After intensive wash with PBS, myotubes were fixed and imaged with confocal microscopy. Scale bars, 10 μ m.

Figure 7. Both PLD1 and PLD2 contribute to the macropinosome formation in OS-treated myotubes. (A) Kinetic distribution of PABD-GFP and Dyn2-mCherry in an OS-treated myotube. Boxed areas were magnified and

adjusted for lower green fluorescence intensity to better observe the macropinosome on top of a nucleus. (B, C) Distribution of PLD1 and PLD2 in OS-induced macropinosomes. C2C12-derived myotubes expressing GFP-PLD1, GFP-PLD2 and Dyn2-mCherry were treated with isotonic buffer or OS and followed by fixation and image acquisition with confocal microscopy. Single focal images were shown with scale bars of 10 μ m. (D) Effects of PLD inhibitors on OS-induced macropinosome formation. Myotubes were pre-treated with indicated inhibitors for 10 min followed by OS that also contained the indicated inhibitors. Phase contrast images of treated cells were acquired with inverted microscopy and the phenotypes were divided into three categories: crowded (macropinosome with diameter > 2 μ m), intermediate and sparse as shown in graph. Data from three independent experiments with $n > 100$ myotubes for each condition were compared to DMSO-treated control. #, $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$.

Figure 8. Model of tension drop-induced lipid raft disruption and macropinocytosis. Under normal tension, lipid phase separation results in lipid-ordered domain formation thus segregates PLD2 from its activator PI(4,5)P₂ as well as its substrate PC (upper panel). When membrane tension is reduced suddenly, the decreased line tension would favor smaller lipid microdomains thus allow PLD2 to access its activator and substrate. The production of PA on plasma membrane leads to PI4P-5 kinase activation to generate PI(4,5)P₂ and promotes higher PLD activity, actin polymerization, Dyn2 activation and subsequently membrane ruffling and macropinocytosis.

Fig. 1

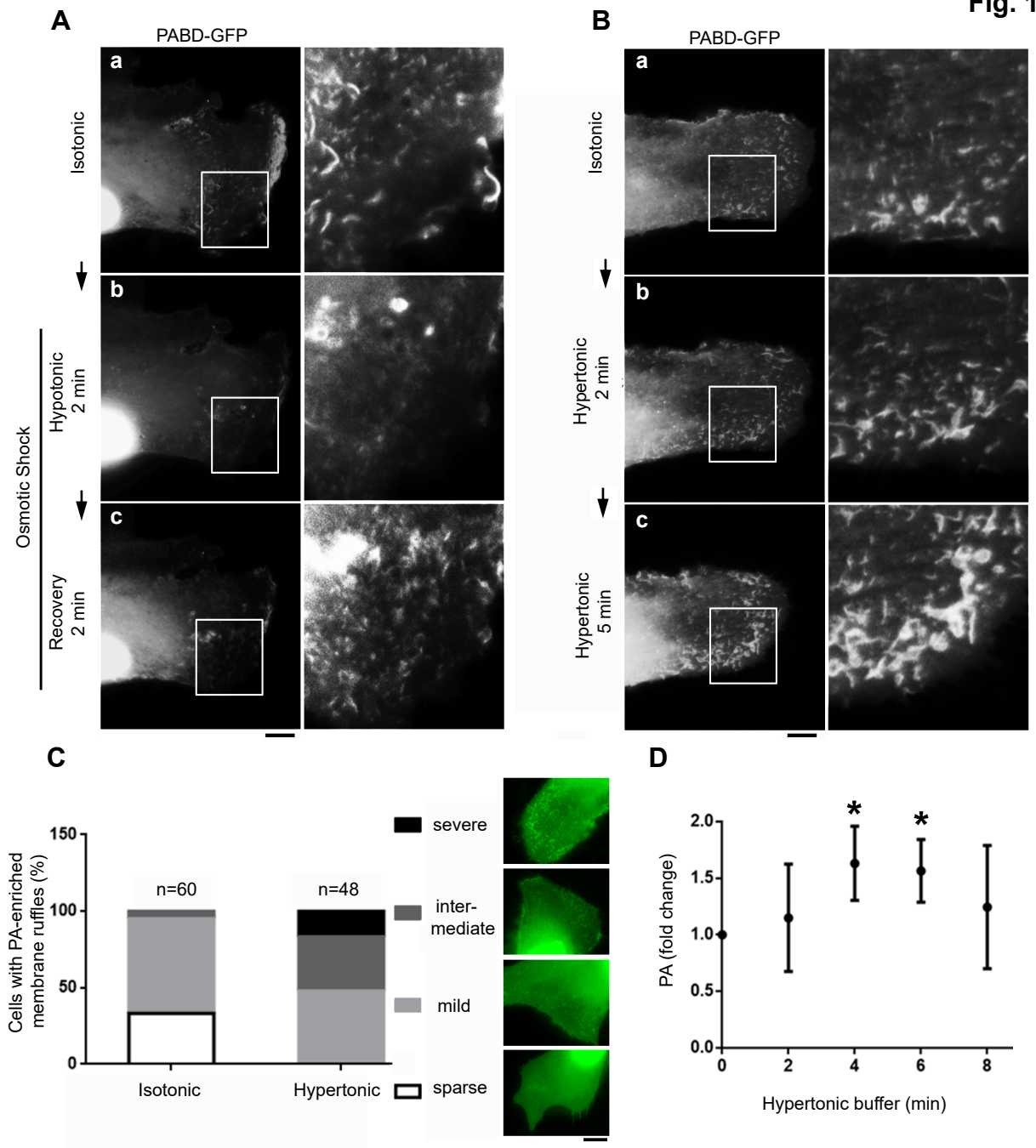
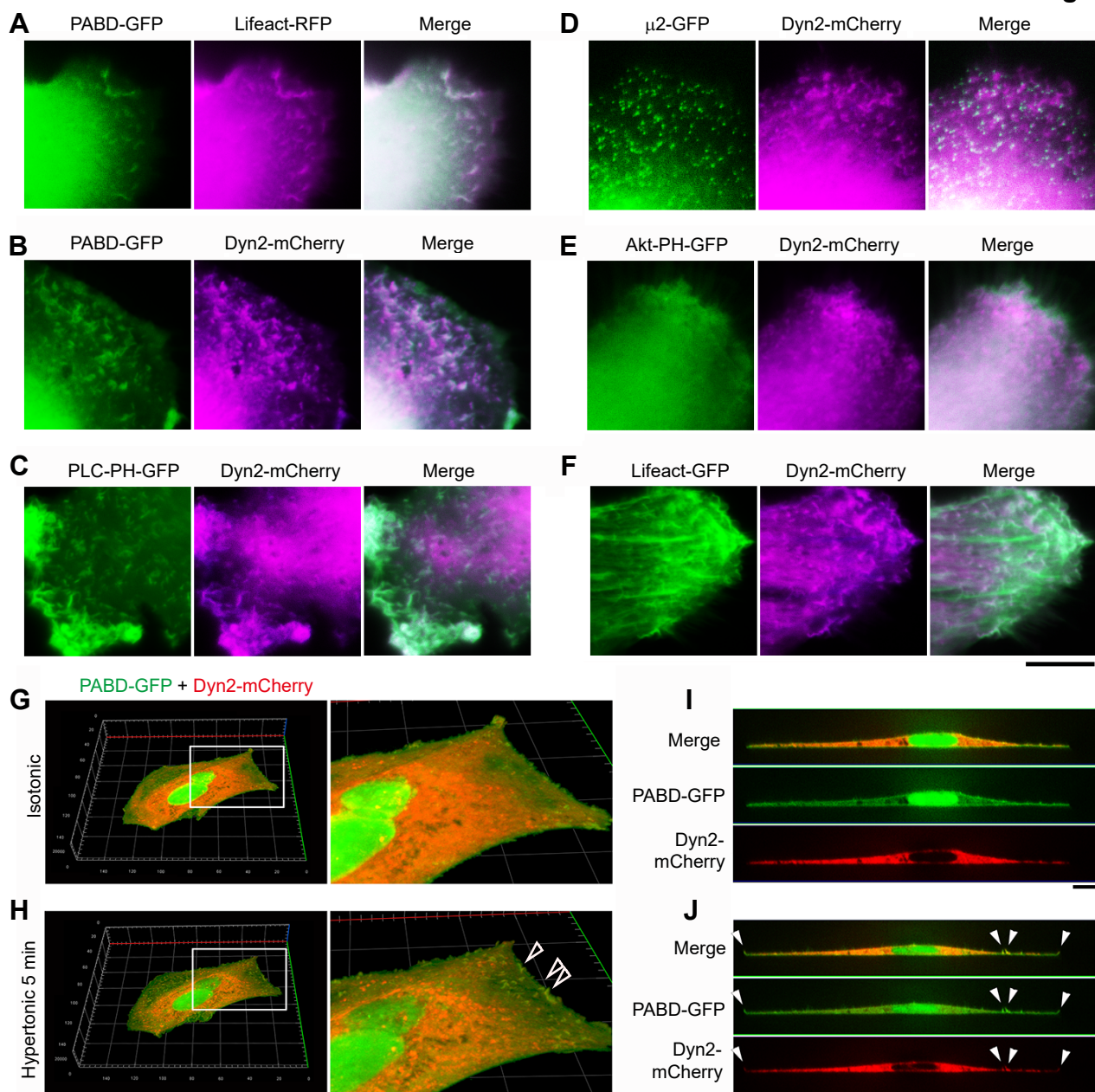


Fig. 2

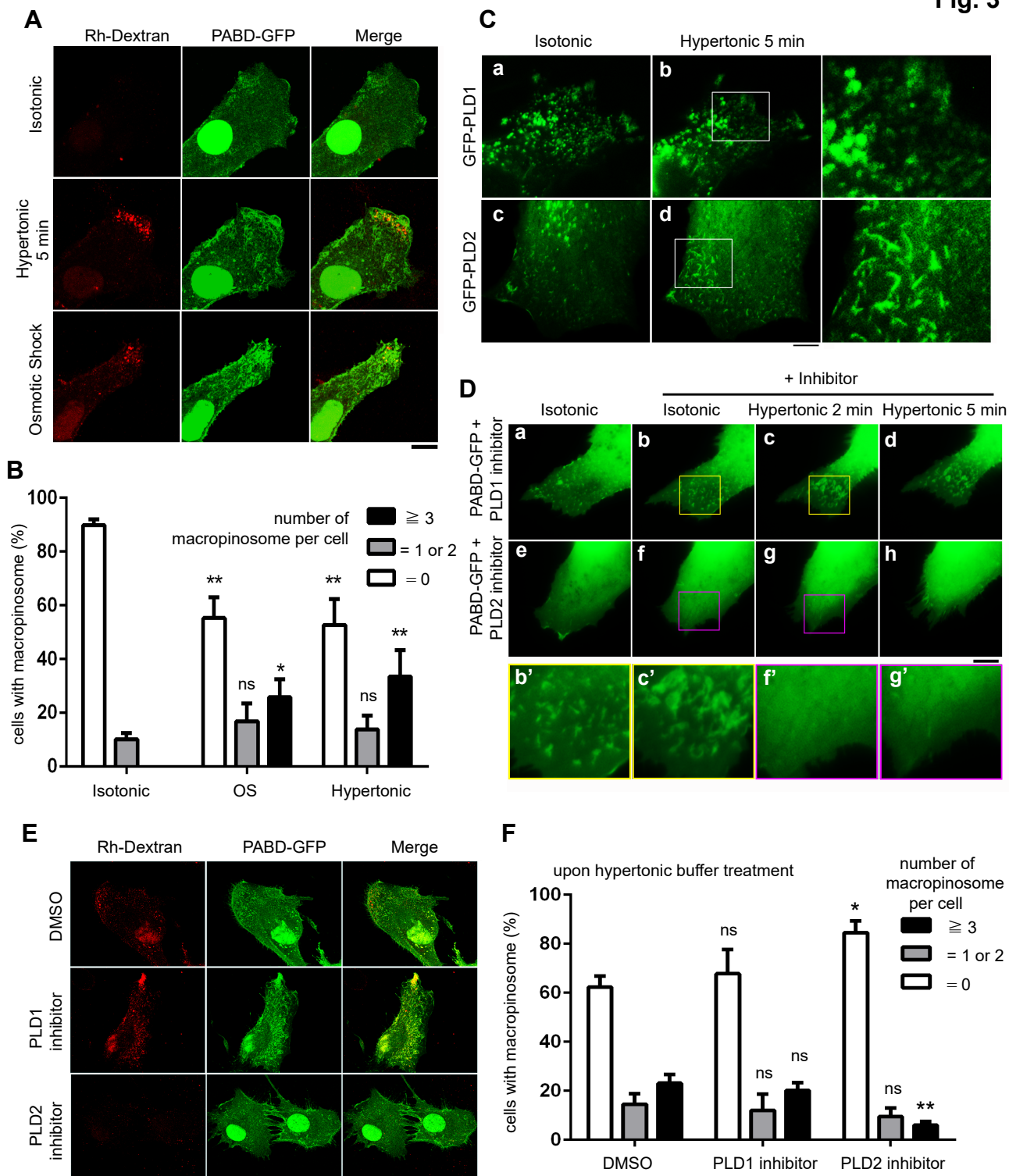


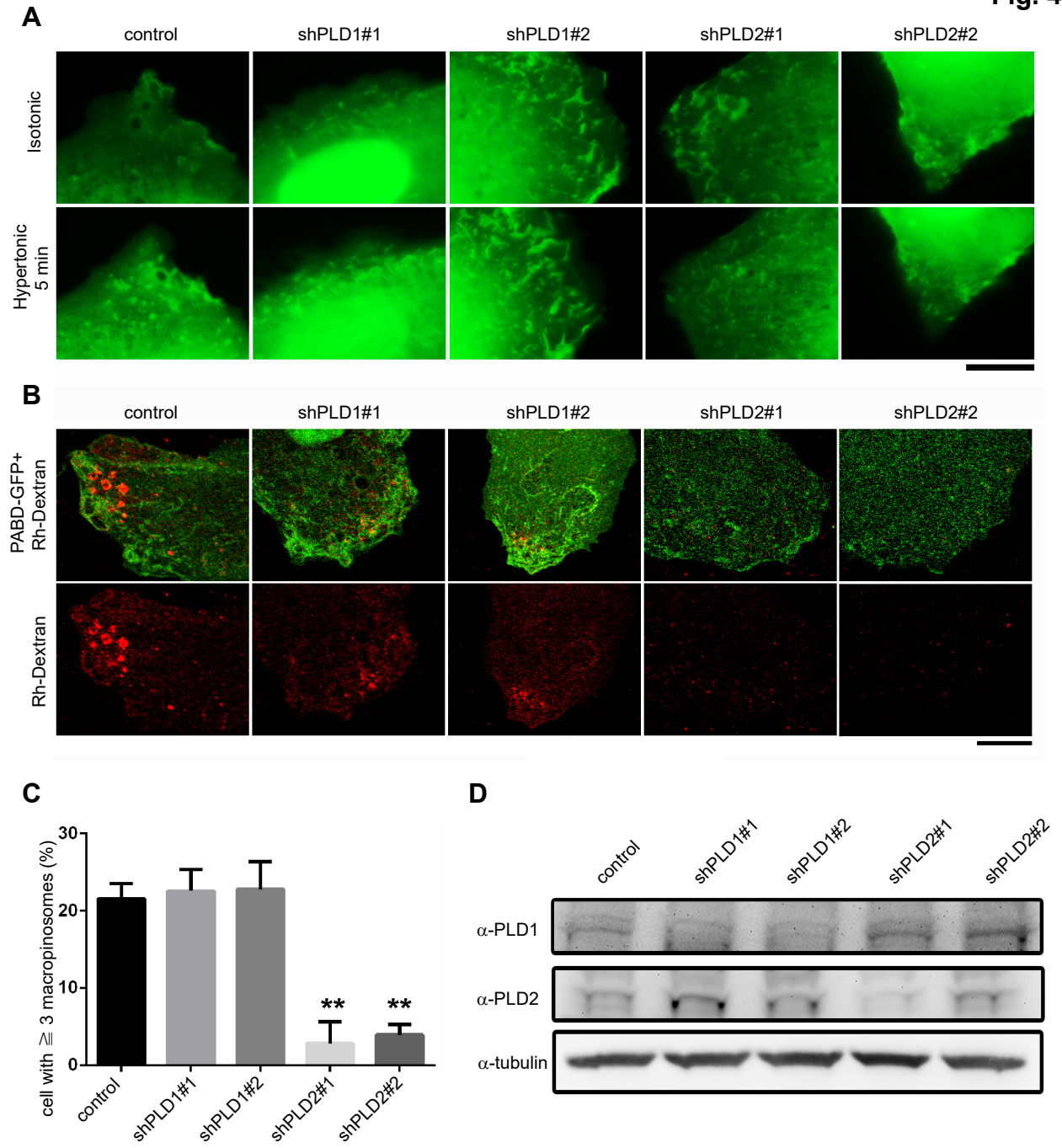
Fig. 4

Fig. 5

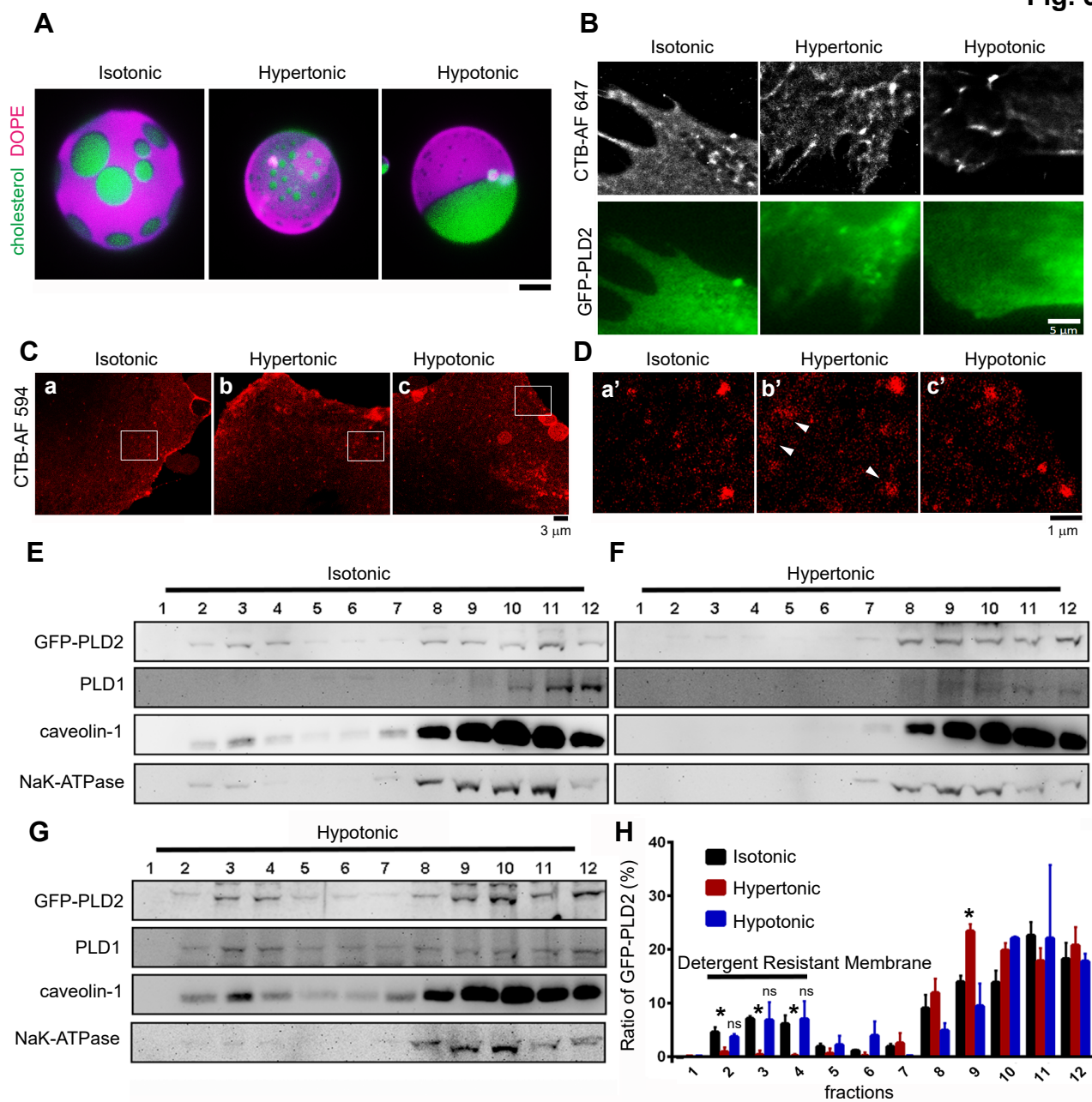


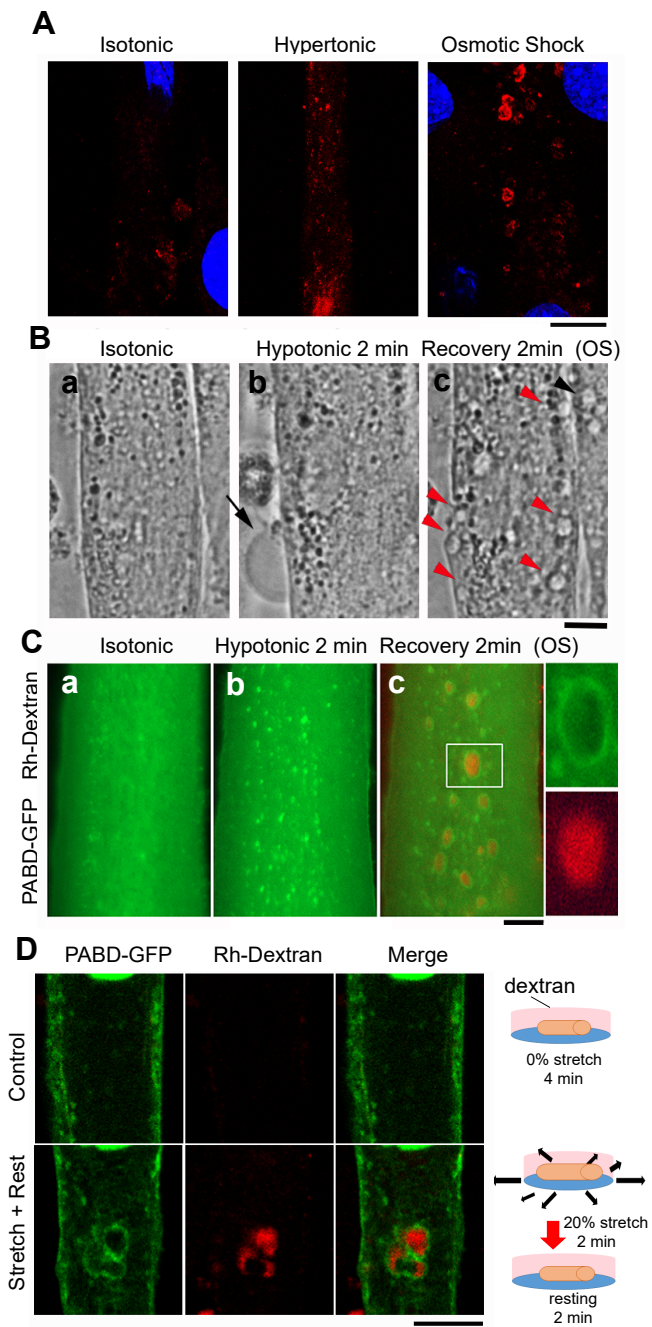
Fig. 6

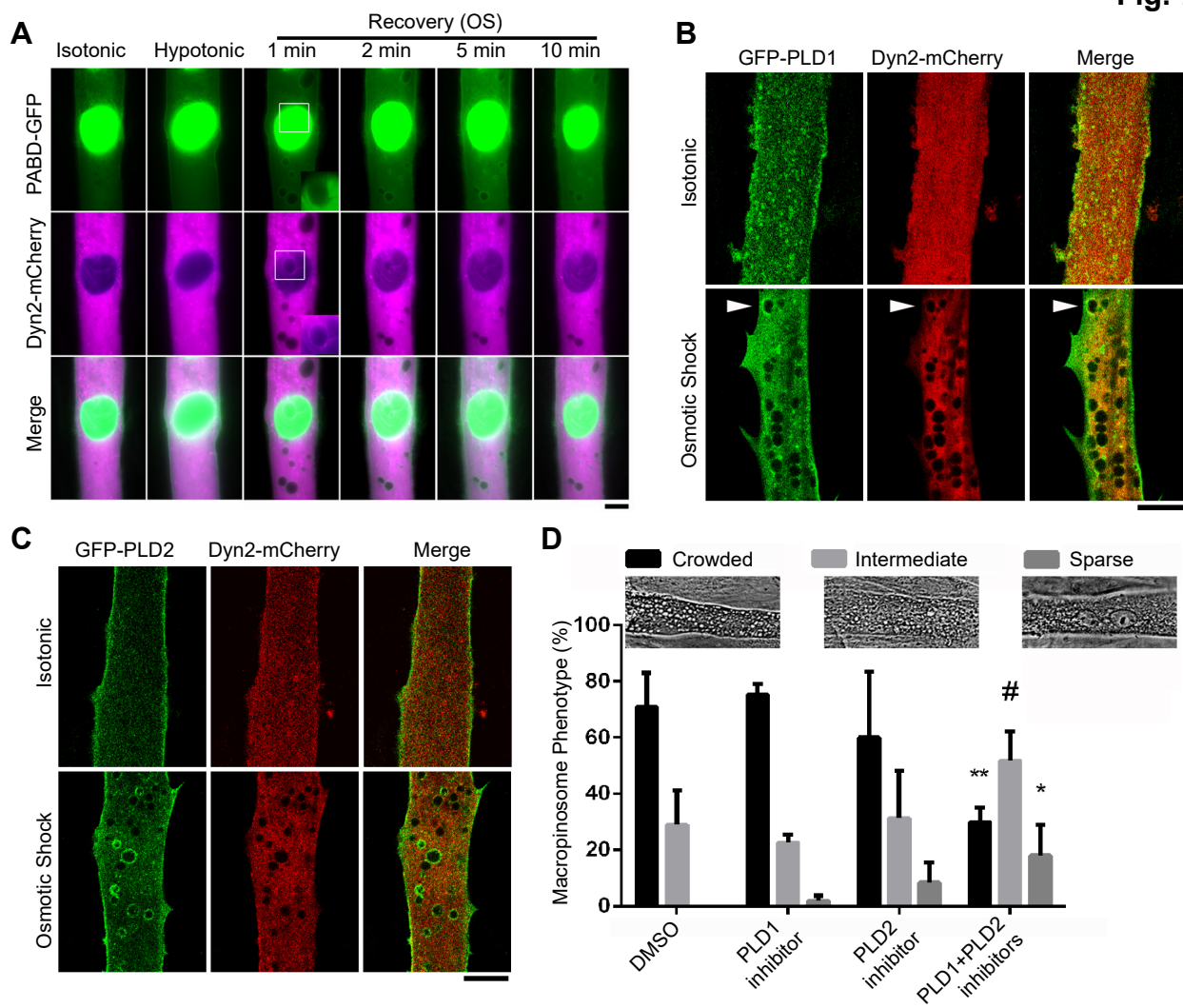
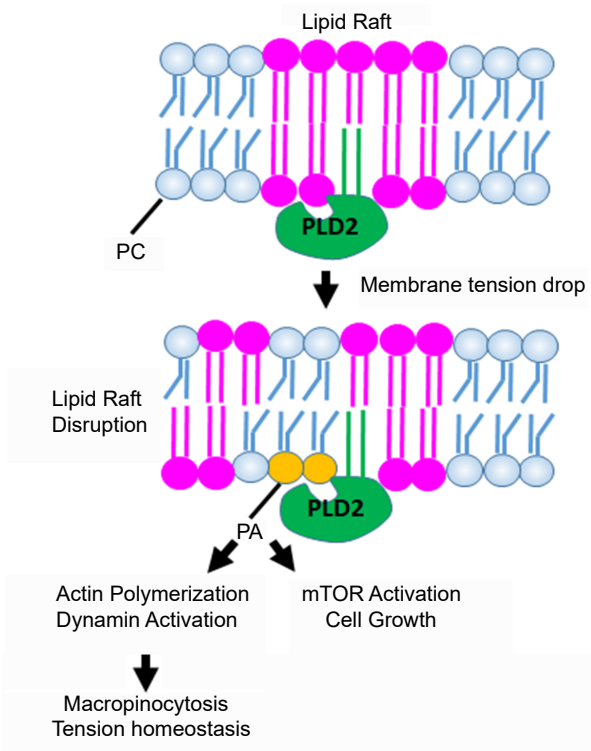
Fig. 7

Fig. 8



SUPPLEMENTARY INFORMATION

Inventory of supplementary information (SI):

- 1. Three supplementary figures (Fig S1 is related to Fig1, Fig S2 is related to Fig 3, Fig S3 is related to Fig 4, Fig S4 is not related to any figure);**
- 2. Three supplementary movies (these movies are related to Fig. 5).**

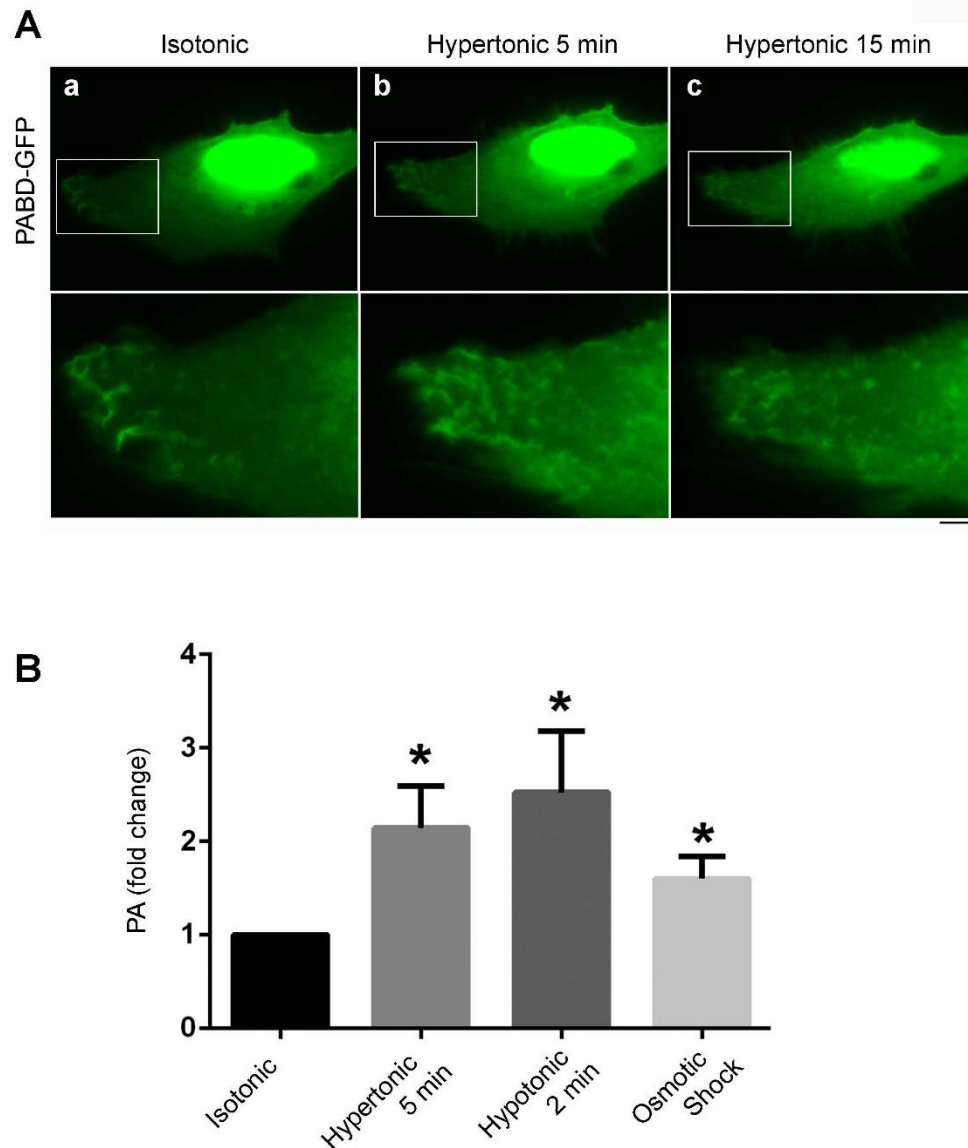


Figure S1. PA distribution and quantification upon tension manipulation.

(A) Distribution of PA was monitored with PABD-GFP in myoblast treated with hypertonic buffer for 5 or 15 min. (B) Effects of different osmotic buffers on cellular PA amount. Myoblasts treated with indicated buffers were harvested and extracted for total lipids. Total PA/protein ratio were compared with the isotonic buffer-treated cells. *, $p < 0.05$. Scale bar, 10 μm .

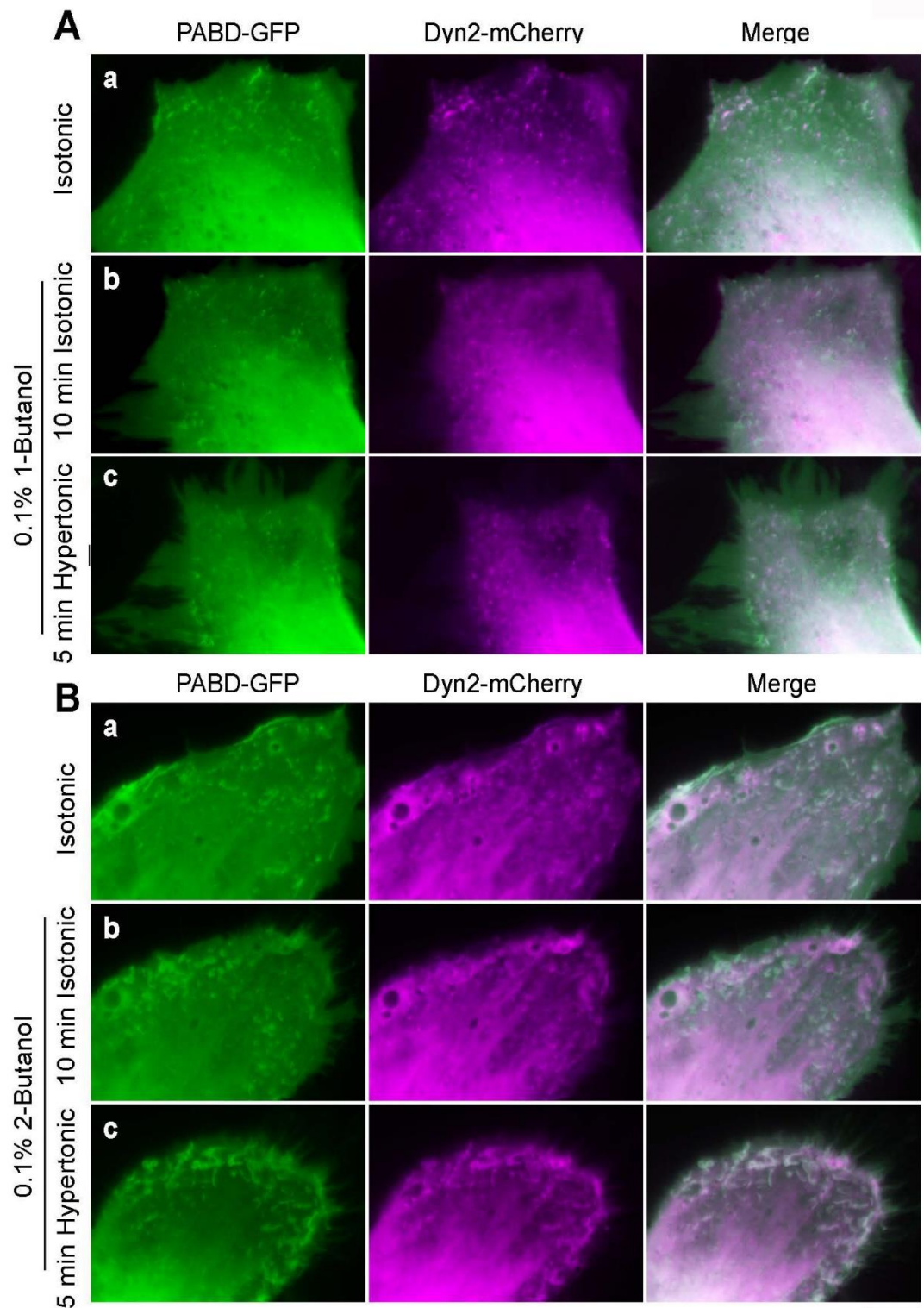


Figure S2. Effects of butanol on tension-induced, PA-rich membrane ruffling. PABD-GFP and Dyn2-mCherry transfected myoblasts were pretreated with 0.1% 1-butanol (A) or 2-butanol (B) in isotonic buffer for 10 min and subjected to hypertonic buffer incubation in the presence of indicated alcohol. Scale bar, 10 μ m.

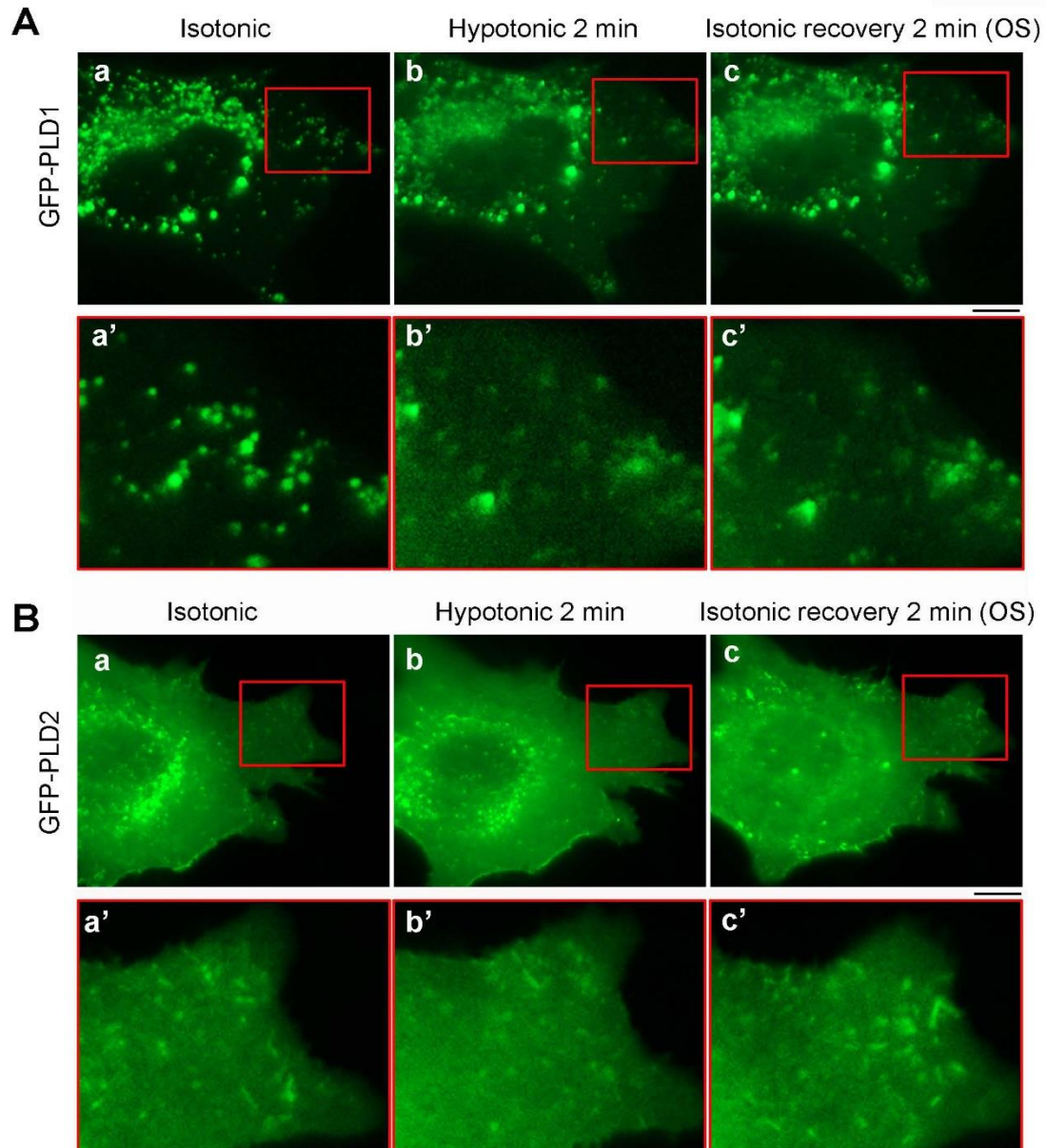


Figure S3. Subcellular distribution of GFP-PLD1 or GFP-PLD2 upon OS treatment. Myoblast expressing GFP-PLD1 or GFP-PLD2 were imaged in isotonic buffer (a), hypotonic buffer (b) and finally in isotonic recovery buffer (c) to monitor the kinetic distribution of these two PLDs. Magnified images of the boxed area are shown in the lower panel. Scale bar, 10 μ m.

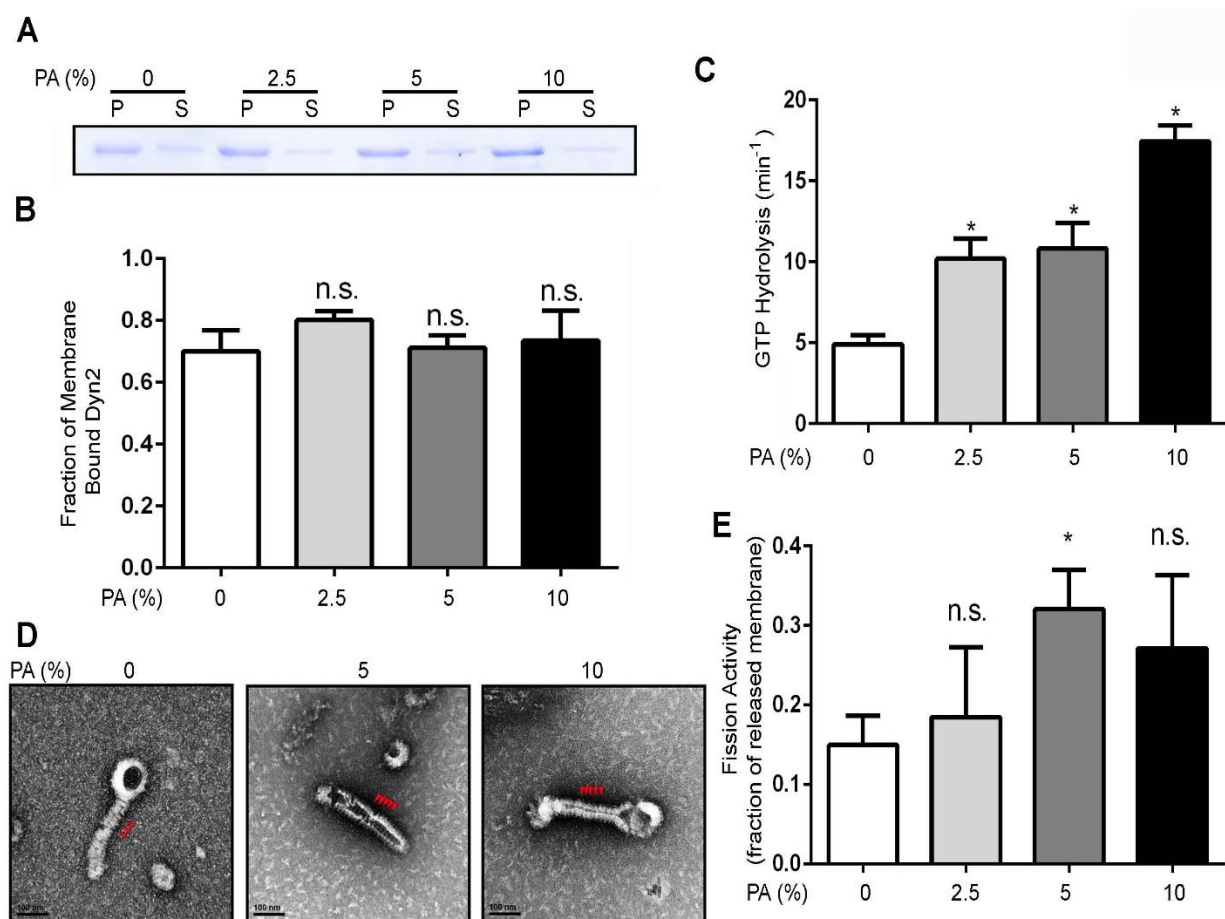


Figure S4. Effects of PA-containing membrane on Dyn2 activity. (A)

Liposome binding assay. 0.5 μM purified human Dyn2 was incubated with 150 μM , 400 nm liposomes of indicated mole concentration of PA at 37°C for 15 min and sedimented into pellet (p) and supernatant (s) fractions by centrifugation. A representative Coomassie blue stained gel of p and s fractions of different percentage of PA-containing liposomes is shown. (B) The fraction of Dyn2 bound to liposomes were quantified with SDS-PAGE electrophoresis, Coomassie blue staining and ImageJ quantification. (C) Lipid-stimulated GTPase activity of Dyn2. 0.5 μM Dyn2 was incubated with 400-nm liposomes containing different PA concentration in the presence of 1 mM GTP at 37 °C. Released Pi was determined using a colorimetric malachite green assay. (D) Electron micrographs of Dyn2 assembled onto 100-nm liposomes with varying concentrations of PA. Red arrowheads indicate Dyn2 spirals. (E) Membrane fission activity of Dyn2. 0.5 μM Dyn2 was incubated with SUPER templates containing varying amounts of PA for 30 min in the presence of GTP. Membrane fission was measured by the release of fluorescently labeled vesicles into the supernatant after centrifugation of the SUPER templates. The data was normalized to its total lipids amount in the template. Data are shown as average

\pm SD (n=3). *, $p < 0.05$.