

# **Alteration of transbilayer phospholipid compositions is involved in cell adhesion, cell spreading, and focal adhesion formation**

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Running title: Expression of phosphatidylcholine flippases inhibits cell spreading

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## **Abstract**

We previously showed that P4-ATPases, ATP10A/ATP8B1, and ATP11A/ATP11C have flippase activities toward phosphatidylcholine (PC), and aminophospholipids (phosphatidylserine [PS] and phosphatidylethanolamine), respectively. Here, we investigate the effect of PC-specific flippases versus aminophospholipid-specific flippases in cell spreading on the extracellular matrix. Expression of PC-flippases, but not PS-flippases, delayed cell adhesion, cell spreading and inhibited formation of focal adhesions. In addition, overexpression of a PS-binding probe that sequesters PS in the cytoplasmic leaflet delayed cell spreading and inhibited formation of focal adhesions. These results suggest that elevation of PC at the cytoplasmic leaflet of the plasma membrane by expression of PC-flippases may reduce the local concentration of PS or phosphoinositides, required for efficient cell adhesion, focal adhesion formation, and cell spreading.

## **Introduction**

Adhesion of cells to the extracellular matrix (ECM) plays a crucial role in regulating cellular functions, such as cell migration, differentiation, proliferation, and survival [1]. Binding of cells to the ECM triggers complex networks of intracellular signaling pathways, leading to spreading and adhesion of cells on the ECM. The connection between the cytoskeleton and the ECM is provided by heterodimeric receptors of the integrin family. In response to extracellular ligand binding, integrins undergo conformational changes to recruit cytoplasmic adaptor proteins, resulting in progressive

formation of highly complex cell–matrix adhesions [2-4]. The assembly of cell–matrix adhesions is based on allosteric conformational changes of integrins by protein–protein as well as protein–lipid interactions [1, 2, 5]. A number of focal adhesion-related proteins, such as vinculin, talin, and kindlin, interact with negatively charged phospholipids in the cytoplasmic leaflet of the plasma membrane to be recruited and activated at focal adhesion sites [6-9]. A polybasic region of Rac1 is able to recognize phosphatidylserine (PS) and is required for Rac1 recruitment to the plasma membrane [10]. PS is also required for Cdc42 recruitment to the polarized plasma membrane in budding yeast [11].

In eukaryotic cells, the lipid bilayer of cellular membranes exhibits asymmetric lipid distribution [12-14]. In the plasma membrane, aminophospholipids, PS and phosphatidylethanolamine (PE), are restricted primarily to the inner/cytoplasmic leaflet, while phosphatidylcholine (PC) and sphingomyelin (SM) are exposed on the cell surface [15-17]. Type IV P-type ATPases (P4-ATPases) are essential for establishing and regulating phospholipid asymmetry in lipid bilayers in eukaryotic cellular membranes [18-22]. The yeast P4-ATPases (phospholipid flippases) Drs2p and Dnf1p/Dnf2p flip PS and PC/PE, respectively [20, 23, 24]. Mammals express 14 P4-ATPases: class 1 (ATP8A1, ATP8A2, ATP8B1, ATP8B2, ATP8B3, and ATP8B4), class 2 (ATP9A and ATP9B), class 5 (ATP10A, ATP10B, and ATP10D), and class 6 (ATP11A, ATP11B, and ATP11C). We recently showed that ATP11A and ATP11C can flip NBD-labeled aminophospholipids, NBD-PS and -PE, and that ATP8B1, ATP8B2, and ATP10A preferentially flip NBD-PC at the plasma membrane [25-27].

In this study, we extend our previous observation that exogenous ATP10A expression causes changes in cell shape and cell size, and inhibits cell adhesion on the ECM [26]. We found that expression of PC-specific flippases, ATP8B1 and ATP10A, but not PS/PE-specific flippases, ATP11A or ATP11C, inhibited cell adhesion, cell spreading, and formation of focal adhesions on the ECM. Importantly, sequestration of PS in the cytoplasmic leaflet of the plasma membrane by exogenous expression of a PS-specific probe also inhibited cell spreading and formation of focal adhesions.

## **Materials and Methods**

**Plasmids** — Expression vectors for C-terminally HA-tagged P4-ATPases were constructed as described previously [28]. A cDNA fragment of the lactadherin C2 domain (LactC2) was kindly provided by Hiroyuki Arai (The University of Tokyo, Japan) and subcloned into the pEGFP vector (Clontech) as described previously [29]. Lentiviral vectors, pRRLsinPPT, pRSV-REV, pMD2.g, and pMDL/pRRE [30] were kindly provided by Peter McPherson (McGill University, Canada). A fragment of EGFP-LactC2 or EGFP is subcloned into pRRLsinPPT vector.

**Antibodies and reagents** — Sources of antibodies used in the present study were as follows: monoclonal mouse anti-paxillin (clone 349), BD Biosciences; monoclonal rat anti-HA (3F10), Roche Applied Science; Alexa Fluor–conjugated secondary antibodies, Molecular Probes; Cy3-conjugated

secondary antibodies, Jackson ImmunoResearch Laboratories. Alexa Fluor–conjugated phalloidin was purchased from Molecular Probes. Fibronectin was purchased from Sigma-Aldrich.

***Cell culture and immunofluorescence analysis*** — HeLa cells were maintained in Minimum Essential Medium Eagle (MEM) (Nacalai Tesque) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and non-essential amino acids (Nacalai Tesque). Cells stably expressing HA-tagged P4-ATPases have been obtained as described previously [25, 26].

To obtain EGFP-LactC2 or EGFP expressing cells, pRRLsinPPT-EGFP-LactC2 or pRRLsinPPT-EGFP was transfected into HEK293T cells using Polyethylenimine Max (Polysciences) together with the packaging plasmids (pRSV-REV, pMD2.g, and pMDL/pRRRE). The resultant lentiviruses were concentrated as described previously [29] and then used to infect HeLa cells. The infected cells were selected in medium containing 1 mg/ml of G418 (Nacalai Tesque). A mixed population of drug-resistant cells was used for the assay. Immunofluorescence staining was performed as described previously [31, 32] and the stained cells were observed using an Axiovert 200MAT microscope (Carl Zeiss).

***Cell adhesion and spreading assay*** — HeLa cells were detached from dishes in PBS containing 5 mM EDTA, and harvested by centrifugation. The cells were washed and resuspended in complete growth medium, plated onto 24-well plates ( $1 \times 10^5$  cells per well), and incubated at 37°C in 5% CO<sub>2</sub> for the indicated times. The same number of cells was removed, and DNA contents were measured using a Qubit fluorometer (Life Technologies). After incubation at 37°C, the cells were fixed with 96% ethanol and stained with 1% crystal violet in 10% ethanol at room temperature. After washing the cells with PBS, the stain was extracted using 1% Triton X-100 and processed for measurement of absorbance at 570 nm. In order to achieve a more accurate assessment, the absorbance was normalized to the DNA content of the seeded cells, even though the same numbers of cells were seeded.

For cell-spreading assay, cells were harvested as described above, washed with serum-free MEM, and seeded onto fibronectin (50 µg/ml)- or FBS (as a mixture of extracellular matrix)-coated coverslips. After incubation at 37°C in 5% CO<sub>2</sub> for indicated times, cells were fixed with 3% PFA and subjected to immunofluorescence analysis. Alexa Fluor–conjugated phalloidin was added during incubation with secondary antibody. Immunofluorescence staining was performed as described previously [31, 32] and observed using an Axiovert 200MAT microscope (Carl Zeiss).

To obtain quantitative data of the extent of cell spreading, cells were stained with Alexa Fluor–conjugated phalloidin, randomly chosen fields were acquired, and cell areas were measured with the MetaMorph software (Molecular Devices). To obtain quantitative data of the number of focal adhesions, cells were stained with anti-paxillin antibody, randomly chosen fields were acquired, and the images were analyzed using an image processing method for focal adhesions by ImageJ software [33].

The image processing method was modified and optimized to identify focal adhesions. In order to increase the cell numbers to be counted, the percentage of cells with more than ten focal adhesions is shown as bar graphs.

## Results and Discussion

### Expression of PC-flippase but not PS-flippase delayed cell adhesion, cell spreading, and focal adhesion formation.

Previously, we demonstrated that ATP10A and ATP8B1 preferentially translocate NBD-labeled PC (NBD-PC), and ATP11A and ATP11C specifically translocate NBD-PS and NBD-PE from the outer/exoplasmic to inner/cytoplasmic leaflet of the plasma membrane [25-27]. The NBD-PC flippase activity of ATP10A is higher than that of ATP8B1 [26], and ATP11C prefers NBD-PS to NBD-PE [25, 27]. We also showed that cell adhesion was delayed in cells stably expressing ATP10A but not its ATPase-deficient mutant, ATP10A(E203Q) [26]. We further wondered whether cell adhesion is affected by expression of PS-specific P4-ATPase. As shown in Figure 1, adhesion of ATP10A-expressing cells was delayed as described previously. By contrast, adhesion of ATP10A(E203Q)-, ATP11A-, or ATP11A(E186Q)-expressing cells was not delayed, but instead slightly accelerated at an early time point (Figure 1, 15 min). Thus, an increase in PC-flipping activity leads to a delay in cell adhesion, while increased PS-flipping activity is unlikely to affect cell adhesion.

Next, we examined cell spreading on FBS- or fibronectin-coated coverslips. To this end, cells stably expressing P4-ATPases were detached from dishes by EDTA treatment, resuspended in serum-free medium, and seeded onto FBS- or fibronectin-coated coverslips. We made use of FBS as a mixture of extracellular matrix. As shown in Figure 2, cells stably expressing a PC-flippase (ATP8B1 or ATP10A) spread more slowly than control cells on FBS-coated coverslips; the cell size was therefore smaller than that of control cells at 60, 120, and 180 min points. At 120 and 180 min points, control cells extended evenly on FBS-coated coverslips, retaining a round shape, while PC-flippase-expressing cells were irregularly extended. The early filopodia formation of ATP8B1- or ATP10A-expressing cells was comparable to that of control cells (Figure 2A, right panels). Cells expressing a PS-flippase (ATP11A or ATP11C) were comparable to control cells not only in early filopodia formation (Figure 2A), but also cell shape during the cell spreading period (Figure 2B, C, and D). We quantified the area of cells expressing PC-flippases and PS-flippases at 60 min (Figure 2E-I) and 120 min (Figure 2J-N) points. The frequency distribution of cell areas revealed that the population of small cells substantially increased upon expression of PC-flippases (Figure 2F, G, K, and L) but not PS-flippases (Figure 2H, M, I, and N). The population of larger cells slightly increased upon expression of PS-flippases (Figure 2H and I), and especially at later time point upon expression of ATP11C (Figure 2N). Therefore, cells stably expressing PC-flippases spread more slowly than control cells and cells expressing PS-flippases become slightly larger than control cells.

Next, P4-ATPase-expressing cells were allowed to spread on fibronectin-coated coverslips. In this experiment, cells expressing ATP10A and ATP11A are used as representatives of PC-flippases and PS-flippases, respectively. Moreover, cells expressing ATP10A(E203Q) are used to examine whether the delay in spreading by expression of ATP10A is due to the lack of its PC-flippase activity. Cells

expressing ATP10A exhibited delayed cell spreading and thus were smaller than control cells at 60, 120, and 180 min points (Figure 3B, C, and D), consistent with our previous study [26]. We quantitated areas of cells expressing ATP10A, ATP10A(E203Q), and ATP11A at 120 min point on fibronectin-coated coverslips (Figure 3E–H) and found that the population of small cells substantially increased only in cells expressing ATP10A. Cells on fibronectin formed remarkable focal adhesions during spreading (Figure 3B–D, control) as compared with cells on FBS-coated coverslips (Figure 2B–D, control). The number of focal adhesions as revealed by immunostaining for paxillin largely decreased in ATP10A-expressing cells (Figure 3B–D). In contrast, cells expressing ATP10A(E203Q) or ATP11A were comparable to control cells in cell spreading and focal adhesion formation. We quantitated the number of focal adhesions and showed the percentage of cells with more than ten focal adhesions (Figure 3I). The extent of focal adhesion formation substantially decreased in cells expressing ATP10A, but not ATP10A(E203Q), indicating that the decrease of focal adhesions is ascribed to enhanced PC-flipping activity (Figure I). The extent of focal adhesion formation was not substantially affected by ATP11A expression (Figure 3I). We also investigated the formation of focal adhesions in cells stably expressing ATP8B1 (PC-flippase) and ATP11C (PS-flippase) on fibronectin (Figure 3J). The extent of focal adhesion formation decreased in cells expressing ATP8B1, although to a lesser extent as compared with that in ATP10A-expressing cells (Figure 3J). It might be ascribed to higher PC-flipping activity observed in ATP10A-expressing cells than that of ATP8B1-expressing cells [25, 26]. Cells expressing ATP11C were comparable to control cells in focal adhesion formation (Figure 3J), although cell spreading slightly increased in ATP11C-expressing cells (Figure 2I and N). These results indicate that an increment in PC flow from the exoplasmic to the cytoplasmic leaflet and/or a decrease in PC content in the exoplasmic leaflet by expression of PC-flippases suppresses cell adhesion, cell spreading, and focal adhesion formation, but not filopodia formation. Although expression of PS-flippases did not substantially affect cell adhesion, cell spreading, or focal adhesion formation, the effect of PS-flippase expression might be underestimated as PS is sparsely distributed in the exoplasmic leaflet at steady state [17]. In contrast, since PC is abundant in the exoplasmic leaflet, expression of PC-flippases may perturb the dynamic equilibrium of transbilayer lipid compositions.

### **Exogenous expression of EGFP-LactC2 inhibits cell spreading and decreases focal adhesions**

We considered two possible reasons why expression of PC-flippases suppresses cell adhesion, cell spreading, and focal adhesion formation: One is that enhanced flow of PC to the cytoplasmic leaflet increases the volume ratio of lipids in the inner to outer leaflet at the plasma membrane and thereby makes cells difficult to extend outward; the other possibility is that the increased PC (a neutral phospholipid) content reduces the local concentration of negatively charged phospholipids, such as PS or phosphoinositides in the cytoplasmic leaflet. Phosphoinositides play critical roles in remodeling the actin cytoskeleton for cell adhesion, spreading, and migration [34–37]. PS is required for recruitment of

Cdc42 to polarized bud tips in budding yeast [11, 38] and for recruitment of Rac1 to the plasma membrane for membrane ruffling and cell migration [10]. However, it has not been shown that PS is required for cell spreading and/or focal adhesion formation. We therefore asked whether PS is involved in the cellular processes. To this end, we exogenously expressed EGFP-tagged lactadherin C2 domain (LactC2), which specifically binds to PS [39], with the intent to sequester PS in the cytoplasmic leaflet. As shown in Figure 4A and B, exogenous expression of EGFP-LactC2, but not EGFP as a negative control, caused a delay in cell spreading on fibronectin-coated coverslips, and thereby an increase in the population of small cells. Moreover, formation of focal adhesions was suppressed by exogenous expression of EGFP-LactC2, but not EGFP, indicating that PS in the cytoplasmic leaflet is required for proper cell spreading and focal adhesion formation (Figure 4C).

Taken together, increased PC flow from the exoplasmic to cytoplasmic leaflet may reduce, at least in part, the local concentration of negatively charged phospholipids, including PS and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), required for cell adhesion and spreading by recruiting and activating focal adhesion-related proteins, such as vinculin, talin, and kindlin, and by regulating actin polymerization and remodeling. These data revealed that appropriate regulation of lipid composition in the lipid bilayer as well as production or concentration of specific phospholipids in the cytoplasmic leaflet, is indispensable for cell adhesion and spreading. In budding yeast, PS is concentrated at the incipient bud sites during cell cycle and is enriched in the tips of mating projections in response to mating factors [11]. The polar localization of PS is required for the recruitment of Cdc42, which is an essential factor for cell polarity, and PE/PC-flippases (Dnf1 and Dnf2) play a role in the dissociation of Cdc42 from the plasma membrane probably by reducing the interaction between C-terminal cationic region of Cdc42 and negatively charged PS in the cytoplasmic leaflet [38, 40]. Therefore, spatiotemporal changes in the lipid composition achieved by flippases may play a regulatory role in the plasma membrane polarization for many cellular functions such as cell migration, cytokinesis, and neurite outgrowth. It is tempting to speculate that the initial cue and maintenance of the plasma membrane polarization might be derived from the regulation of transbilayer lipid composition changes. It also raises an interesting question of how the activity of flippases is regulated in an appropriate time and place.

## References

1. Kim, C., Ye, F. & Ginsberg, M. H. (2011) Regulation of integrin activation, *Annu Rev Cell Develop Biol* **27**, 321-45.
2. Iwamoto, D. V. & Calderwood, D. A. (2015) Regulation of integrin-mediated adhesions, *Curr Opin Cell Biol* **36**, 41-7.
3. Wehrle-Haller, B. (2012) Assembly and disassembly of cell matrix adhesions, *Curr Opin Cell Biol* **24**, 569-581.
4. Banno, A. & Ginsberg, M. H. (2008) Integrin activation, *Biochem Soc Trans* **36**, 229-34.
5. Wehrle-Haller, B. (2012) Structure and function of focal adhesions, *Curr Opin Cell Biol* **24**, 116-124.
6. Johnson, R. P., Niggli, V., Durrer, P. & Craig, S. W. (1998) A conserved motif in the tail domain of vinculin mediates association with and insertion into acidic phospholipid bilayers, *Biochemistry* **37**, 10211-22.
7. Ziegler, W. H., Liddington, R. C. & Critchley, D. R. (2006) The structure and regulation of vinculin, *Trends Cell Biol* **16**, 453-60.
8. Palmer, S. M., Playford, M. P., Craig, S. W., Schaller, M. D. & Campbell, S. L. (2009) Lipid Binding to the Tail Domain of Vinculin: SPECIFICITY AND THE ROLE OF THE N AND C TERMINI, *J Biol Chem* **284**, 7223-7231.
9. Calderwood, D. A., Campbell, I. D. & Critchley, D. R. (2013) Talins and kindlins: partners in integrin-mediated adhesion, *Nat Rev Mol Cell Biol* **14**, 503-17.
10. Finkielstein, C. V., Overduin, M. & Capelluto, D. G. S. (2006) Cell Migration and Signaling Specificity Is Determined by the Phosphatidylserine Recognition Motif of Rac1, *J Biol Chem* **281**, 27317-27326.
11. Fairn, G. D., Hermansson, M., Somerharju, P. & Grinstein, S. (2011) Phosphatidylserine is polarized and required for proper Cdc42 localization and for development of cell polarity, *Nat Cell Biol* **13**, 1424-1430.
12. Op den Kamp, J. A. (1979) Lipid asymmetry in membranes, *Annu Rev Biophys* **48**, 47-71.
13. van Meer, G., Voelker, D. R. & Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nat Rev Mol Cell Biol* **9**, 112-124.
14. Leventis, P. A. & Grinstein, S. (2010) The distribution and function of phosphatidylserine in cellular membranes, *Annu Rev Biophys* **39**, 407-27.
15. Schrier, S. L., Zachowski, A. & Devaux, P. F. (1992) Mechanisms of amphipath-induced stomatocytosis in human erythrocytes, *Blood* **79**, 782-6.
16. Zwaal, R. F. & Schroit, A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, *Blood* **89**, 1121-32.
17. Murate, M., Abe, M., Kasahara, K., Iwabuchi, K., Umeda, M. & Kobayashi, T. (2015)



Transbilayer distribution of lipids at nano scale, *J Cell Sci* **128**, 1627-38.

18. Tang, X., Halleck, M. S., Schlegel, R. A. & Williamson, P. (1996) A subfamily of P-type ATPases with aminophospholipid transporting activity, *Science* **272**, 1495-7.

19. Daleke, D. L. (2003) Regulation of transbilayer plasma membrane phospholipid asymmetry, *J Lipid Res* **44**, 233-42.

20. Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G. & Holthuis, J. C. M. (2003) Drs2p-related P-type ATPases Dnf1p and Dnf2p Are Required for Phospholipid Translocation across the Yeast Plasma Membrane and Serve a Role in Endocytosis, *Mol Biol Cell* **14**, 1240-1254.

21. Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M. & Tanaka, K. (2004) Cdc50p, a Protein Required for Polarized Growth, Associates with the Drs2p P-Type ATPase Implicated in Phospholipid Translocation in *Saccharomyces cerevisiae*, *Mol Biol Cell* **15**, 3418-3432.

22. Zhou, X. & Graham, T. R. (2009) Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast, *Proc Nat Acad Sci* **106**, 16586-16591.

23. Natarajan, P., Wang, J., Hua, Z. & Graham, T. R. (2004) Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to in vivo function, *Proc Nat Acad Sci* **101**, 10614-10619.

24. Graham, T. R. (2004) Flippases and vesicle-mediated protein transport, *Trends Cell Biol* **14**, 670-7.

25. Takatsu, H., Tanaka, G., Segawa, K., Suzuki, J., Nagata, S., Nakayama, K. & Shin, H. W. (2014) Phospholipid Flippase Activities and Substrate Specificities of Human Type IV P-type ATPases Localized to the Plasma Membrane, *J Biol Chem* **289**, 33543-33556.

26. Naito, T., Takatsu, H., Miyano, R., Takada, N., Nakayama, K. & Shin, H. W. (2015) Phospholipid Flippase ATP10A Translocates Phosphatidylcholine and Is Involved in Plasma Membrane Dynamics, *J Biol Chem* **290**, 15004-17.

27. Takada, N., Takatsu, H., Miyano, R., Nakayama, K. & Shin, H. W. (2015) ATP11C mutation is responsible for the defect in phosphatidylserine uptake in UPS-1 cells, *J Lipid Res* **56**, 2151-7.

28. Takatsu, H., Baba, K., Shima, T., Umino, H., Kato, U., Umeda, M., Nakayama, K. & Shin, H.-W. (2011) ATP9B, a P4-ATPase (a Putative Aminophospholipid Translocase), Localizes to the trans-Golgi Network in a CDC50 Protein-independent Manner, *J Biol Chem* **286**, 38159-38167.

29. Takatsu, H., Katoh, Y., Ueda, T., Waguri, S., Murayama, T., Takahashi, S., Shin, H.-W. & Nakayama, K. (2013) Mitosis-Coupled, Microtubule-Dependent Clustering of Endosomal Vesicles around Centrosomes, *Cell Struct Funct* **38**, 31-41.

30. Thomas, S., Ritter, B., Verbich, D., Sanson, C., Bourbonniere, L., McKinney, R. A. & McPherson, P. S. (2009) Intersectin regulates dendritic spine development and somatodendritic endocytosis but not synaptic vesicle recycling in hippocampal neurons, *J Biol Chem* **284**, 12410-9.

31. Shin, H.-W., Kobayashi, H., Kitamura, M., Waguri, S., Suganuma, T., Uchiyama, Y. & Nakayama,

- K. (2005) Roles of ARFRP1 (ADP-ribosylation factor-related protein 1) in post-Golgi membrane trafficking, *J Cell Sci* **118**, 4039-4048.
32. Shin, H.-W., Morinaga, N., Noda, M. & Nakayama, K. (2004) BIG2, a Guanine Nucleotide Exchange Factor for ADP-Ribosylation Factors: Its Localization to Recycling Endosomes and Implication in the Endosome Integrity, *Mol Biol Cell* **15**, 5283-5294.
33. Horzum, U., Ozdil, B. & Pesen-Okvur, D. (2014) Step-by-step quantitative analysis of focal adhesions, *MethodsX* **1**, 56-9.
34. Sechi, A. S. & Wehland, J. (2000) The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane, *J Cell Sci* **113 Pt 21**, 3685-95.
35. Saarikangas, J., Zhao, H. & Lappalainen, P. (2010) Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides, *Physiol Rev* **90**, 259-89.
36. Suetsugu, S., Kurisu, S. & Takenawa, T. (2014) Dynamic shaping of cellular membranes by phospholipids and membrane-deforming proteins, *Physiol Rev* **94**, 1219-48.
37. Tsujita, K. & Itoh, T. (2015) Phosphoinositides in the regulation of actin cortex and cell migration, *Biochim Biophys Acta* **1851**, 824-31.
38. Das, A., Slaughter, B. D., Unruh, J. R., Bradford, W. D., Alexander, R., Rubinstein, B. & Li, R. (2012) Flippase-mediated phospholipid asymmetry promotes fast Cdc42 recycling in dynamic maintenance of cell polarity, *Nat Cell Biol* **14**, 304-310.
39. Yeung, T., Gilbert, G. E., Shi, J., Silviu, J., Kapus, A. & Grinstein, S. (2008) Membrane Phosphatidylserine Regulates Surface Charge and Protein Localization, *Science* **319**, 210-213.
40. Saito, K., Fujimura-Kamada, K., Hanamatsu, H., Kato, U., Umeda, M., Kozminski, K. G. & Tanaka, K. (2007) Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins, *Dev Cell* **13**, 743-51.

**Footnotes**

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**Abbreviations**

The abbreviations used are: P4-ATPase, type IV P-type ATPase; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; NBD, nitrobenzoxadiazole; ECM; extracellular matrix, LactC2; lactadherin C2 domain.

**Author contributions**

HWS conceived and supervised the study; HWS, RM, and HT designed experiments; RM and TM performed experiments; RM, HWS, HT, and KN analyzed and interpreted data; HWS and KN wrote the manuscript.

## Figure Legends

### Figure 1. Expression of PC-flippase but not PS-flippase delays cell adhesion

The adhesion assay was performed using HeLa cells stably expressing ATP10A-HA, ATP10A(E203Q)-HA, ATP11A-HA, or ATP11A(E186Q)-HA. Cells were seeded onto a plastic dish and incubated for the indicated times. After washing to remove non-adherent cells, adherent cells were fixed and stained with crystal violet. The staining was processed for measurement of absorbance at 570 nm. Graphs are representative of two or three independent experiments, and results display averages from quintuplicates  $\pm$  SD.

### Figure 2. Expression of PC-flippases but not PS-flippases delays cell spreading

HeLa cells stably expressing ATP8B1-HA, ATP10A-HA, ATP11A-HA, or ATP11C-HA were processed for the spreading assay. Cells were seeded onto FBS-coated coverslips and incubated for the indicated times. After fixation and permeabilization, cells were incubated with anti-paxillin antibody followed by Alexa Fluor 555-conjugated anti-mouse antibody and Alexa Fluor 488-conjugated phalloidin (A–D). Bars, 20  $\mu$ m. (E–N) Cell areas were measured by the MetaMorph software, and the frequency distribution of cell areas at 60 and 120 min points are shown. Graphs are representative of at least three independent experiments except ATP8B1.

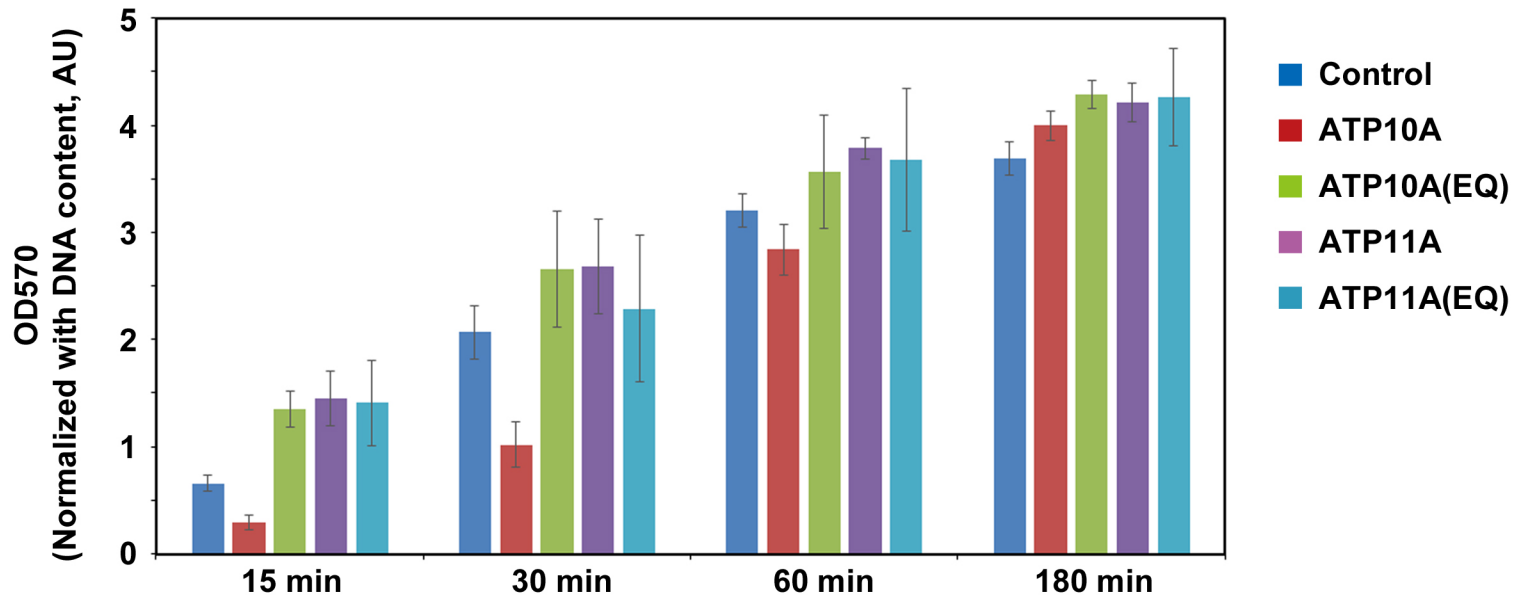
### Figure 3. Expression of PC-flippase but not PS-flippase delays cell spreading and focal adhesion formation on the fibronectin coat

HeLa cells stably expressing ATP10A-HA, ATP10A(E203Q)-HA, or ATP11A-HA were processed for the spreading assay. Cells were seeded onto fibronectin-coated coverslips and incubated for the indicated times. After fixation, immunofluorescence analysis was performed as described in the legend of Figure 2 (A–D). Bars, 20  $\mu$ m. (E–H) Cell areas were measured by the MetaMorph software, and the frequency distribution of cell areas at 120 min is shown. Graphs are representative of at least four independent experiments. (I) Images were processed by ImageJ software to identify focal adhesions, and the percentage of cell numbers with more than ten focal adhesions at 60 min point is shown. (J) HeLa cells stably expressing ATP10A-HA, ATP8B1-HA, or ATP11C-HA were seeded onto fibronectin-coated coverslips and incubated for 60 min. After fixation, immunofluorescence analysis and assessment of focal adhesions were performed as described above. Graphs are representative of at least three independent experiments except ATP8B1. FAs; focal adhesions.

### Figure 4. Expression of EGFP-LactC2 delays cell spreading and focal adhesion formation on the fibronectin coat

HeLa cells were transiently transfected with an expression vector encoding EGFP-tagged LactC2 or EGFP alone. After 24 hr, cells were detached and seeded onto fibronectin-coated coverslips and

incubated for 60 min. After fixation, immunofluorescence analysis was performed as in Figure 2 (A). Bars, 20  $\mu\text{m}$ . (B) Cell areas were measured by the MetaMorph software, and the frequency distribution of cell areas at 60 min is shown. (C) Images were processed by ImageJ software to identify focal adhesions, and the percentage of cell numbers with more than ten focal adhesions at 60 min point is shown.



**Figure 1 Miyano et al.**

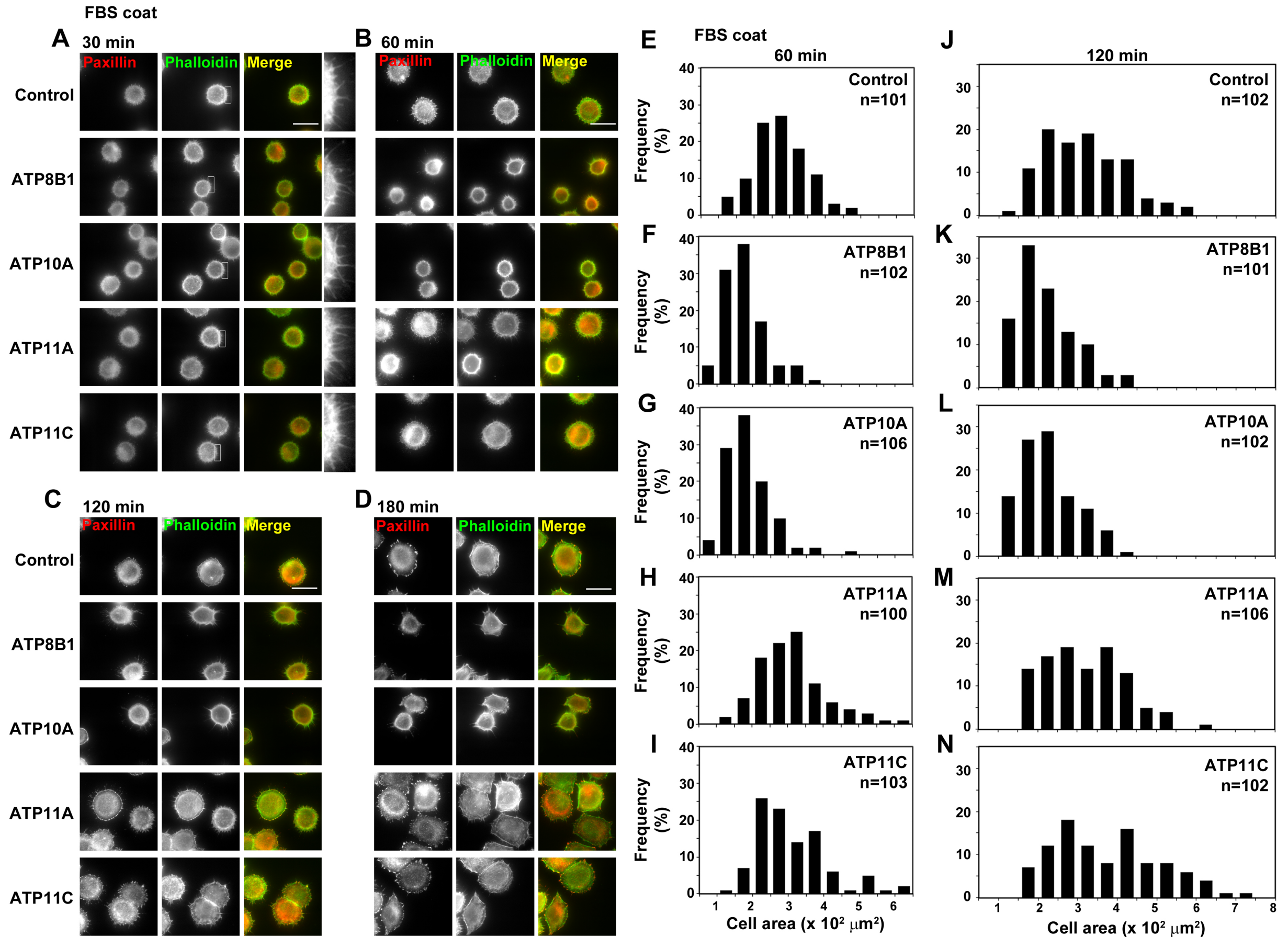


Figure 2 Miyano et al.

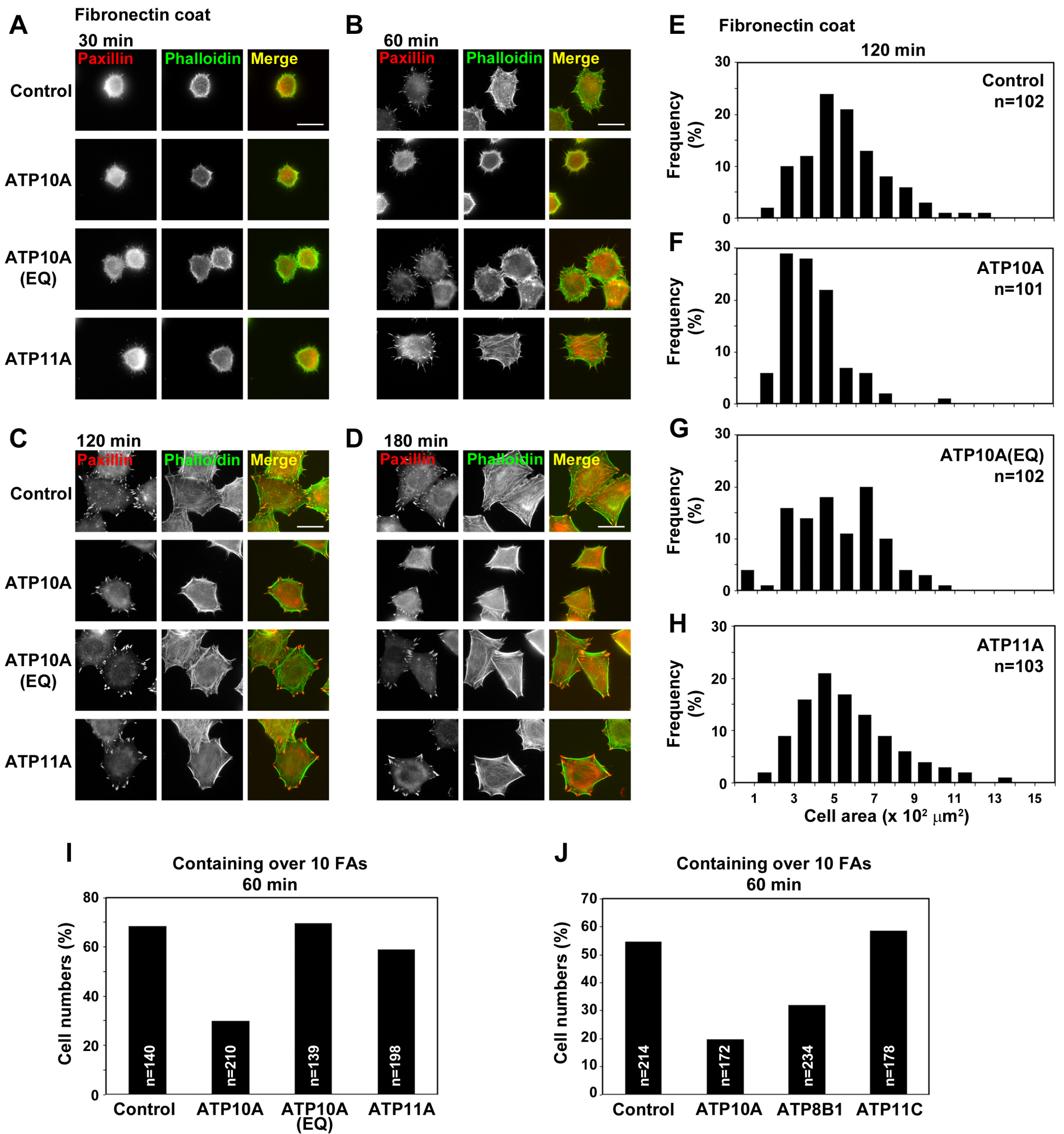


Figure 3 Miyano et al.



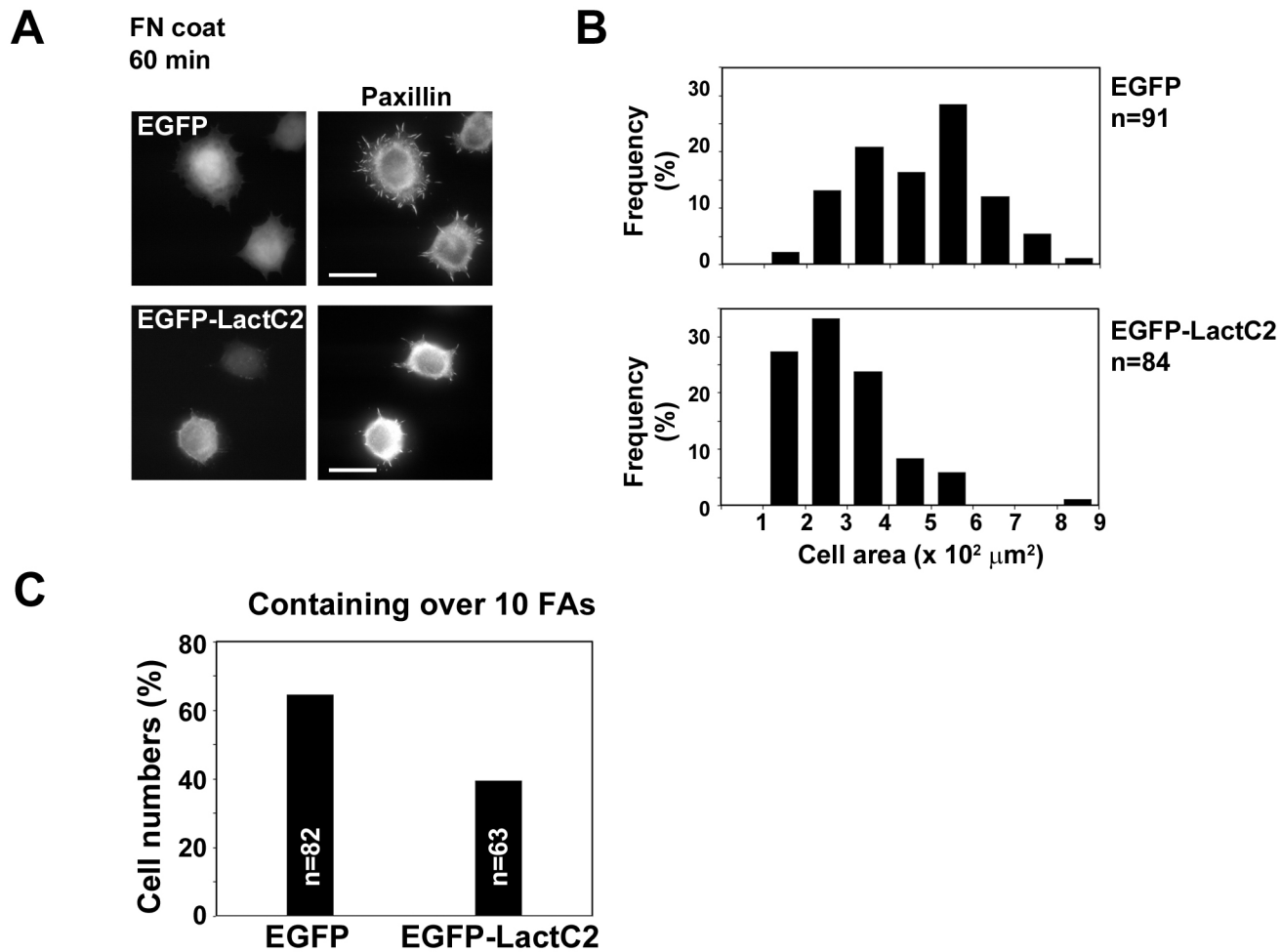


Figure 4 Miyano et al.