Archives of Razi Institute, Vol. 78, No. 1 (2023) 261-267

DOI: 10.22092/ARI.2022.358953.2340

Original Article

Evaluation of the Role of MAP4K4 in Focal Adhesion Dynamics and Regulation of Cell Migration of Breast Cancer Cell Line MDA-MB-231

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Received 7 June 2022; Accepted 10 July 2022
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Abstract

In the migration and metastasis of cancer cells, it is necessary to rotate the focal adhesion (FA). MAP4K4 plays a vital role in the formation of cytoskeletal regeneration, but its role in regulating FA dynamics and cancer cell migration is not well understood. This present aimed to investigate the role of MAP4K4 in regulating FA dynamics and cell migration in the human breast cancer cell line. For this purpose, different variants, including MAP4K4 (wild type), partial active mutation kinase (MAP4K4-T178D), mutant with inactivated or reduced activity kinase (MAP4K4-T178A) and inactive kinase mutation (MAP4K4-K54R) was used in the evaluation. GFP-paxillin was also used as a marker in basal breast cancer cells (MDA-MB-231) in determining FA dynamics. Time-lapse and confocal microscopes were used to record FA dynamics and cell migration. The present study's findings showed that cells expressing MAP4K4-K54R, MAP4K4-T178D and MAP4K4-T178A type slowly down the FA turnover rates and had much larger FAs than those expressing WT MAP4K4 in MDA-MB-231 breast cancer cell line. Furthermore, inhibiting MAP4K4 strongly inhibited FA formation and reduced cell migration speed. In conclusion, MAP4K4 regulates FA dynamics and cancer cell migration, most probably through activating FA proteins and cytoskeleton.

Keywords: cancer cell migration, focal adhesions, MAP4K4, FA dynamics

1. Introduction

Cell migration plays a vital role in the life of multicellular organisms, organizing the nervous system and producing specialized organs and tissues. It is also effective in many biological and physiological processes, such as wound healing (1). In a microscopic examination of cell migration, there are several steps, which include expansion of the anterior edge, adhesion to the sides of the matrix, contraction of the cytoplasm, diffusion from the contact sites, and recycling of membrane receptors from the back to the front of the cell, respectively (2). Focal adhesions (FAs) consist of macromolecules that enable them to interact with extracellular molecules by making successive temporary connections to migrating cells. Extracellular signalling leads to cell assembly and separation processes (3). In order to stretch the internal cytoskeleton forward, which is the first step in the FAs cycle, the lipopodium contains proteins such as tallin, paxillin, vincoline, and integrin, which forms a wide and flat structure that leads to forward cell stretching (4, 5). With the separation of the extracellular matrix from the FA and the proteolysis of the proteins involved, such as calpain and microtubules involved in

the circulation of the FA, the cell movement cycle ends (6, 7).

MAP4K4 is a serine/threonine protein kinase STE20 from the Germinal Center Kinase (GCK) family, which has catalytic activity in the N-terminal region and regulatory activity in the C-terminal region. MAP4K4 is associated with MEKK1 and has the activity of mitogen-activated protein kinases (MAPKs). This protein is an upstream activator of the c-Jun N family of terminal kinases (JNK) and also acts as a mediator in the activity of alpha tumour necrosis factor (TNFα) (8, 9). The amino acid residues of lysine 54 (K54), threonine 181 (T181) and threonine 187 (T187) involved in the regulation of MAP4K4 activity have been found in the N-terminal region in human Map4K4 (Figure 1) (10, 11). Given that there are reports that cells need MAP4K4 to form a cytoskeleton in migrating cells, its exact role is still unknown. One of the effective processes of MAP4K4 is activating integrin signalling pathways. Tallin is the ERM protein family (ERMs) substrate for MAP4K4, which, after phosphorylation, separates the terminal region that interacts with membrane proteins from the carboxyl terminus associated with actin, creating a bond between actin and plasma membrane to induce cell adhesion (12).

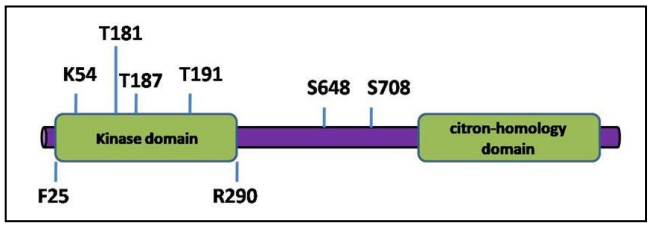

Figure 1. The human MAP4K4 schematic structure consists of an N-terminal kinase domain and a C-terminal citron homology domain.

The present study investigated the requirement for the kinase activity of MAP4K4 in FA dynamics and cancer cell migration. The results identify an essential role of MAP4K4 in FA turnover and cancer cell migration.

2. Materials and Methods

2.1. Cell Line and Cell Culture

The cell line used in this study was an MDA-MB-231 human breast cancer cell obtained from the centre (Manassas®, VA). These cells were grown in a modified medium (DMEM) containing 10% fetal bovine serum and 1% v / v penicillin/streptomycin. The culture medium was incubated at 37 ° C, 90% humidity and 5% CO_2. The Mycoplasma EZPCR diagnostic kit was also used to check for mycoplasma undergrowth at the testing time, according to the instructions.

2.2. Construction of MAP4K4 Kinases

MAP4K4 cloning and vector construction were created by Angela Clerk's laboratory based on the method of Fuller, Edmunds (13).

2.3. Staining and FA Turnover Assay

For staining of transfected cells, the culture medium was washed with PBS and then incubated with 4% paraformaldehyde for 15 minutes at room temperature. Also, additional PFAs were incubated after washing with PBS in PBS containing 0.5% Triton X-100 for 10 minutes at room temperature to increase cell permeability. Finally, these cells were treated for 30 minutes in PBS containing 10% goat serum at room temperature and stained with anti-paxillin antibody (Abcam®) and antibody to the Anti- FLAG epitope (Sigma®) (1:10, 2 µl), 4 µl goat serum (2%) and 200 µl PBS for 1 h, then washed and incubated with 546 Alexa Fluor anti-mouse solution (1:10, 2 µl) (Cell signalling®), 200 µl PBS, 4 µl goat serum (2%) for 1 h in the dark condition. The cells were washed for 10 min and then stained with DAPI. To analyse the number and size of FAs, the fixed cell images were captured digitally using a 100X oil-immersion objective lens. After the brightness & contrast were adjusted, the threshold command was applied to a binary image with two-pixel values, 255 (white) and 0 (black), to ensure only FA 'dots' were selected. Finally, the analysis particles command was applied using the following parameters: size = 0.5-infinity and circularity = 0.00–0.99.

35 mm (Tistle Scientific) culture dishes treated with 2 mg/ml BD Bioscience collagen were used to measure the dynamics of FA 24 hours before transfection, MDA-MB-231 cells (overnight incubation). These cells were subsequently transfected with plasmid DNA (3 µg) consisting of GFP-Paxillin and WT-MAP4K4, MAP4K4-K54R, MAP4K4-T178D or MAP4K4-T178A using the TurboFect™ transfection reagent (Thermo Scientific®) at a ratio of 1:1 (w/v, respectively).

Imaging and monitoring of cells' FA dynamic and the whole lifetime of paxillin assembly and disassembly were performed at 488 nm and 568 nm. (Every 5 seconds for 10 minutes). Imaging was performed using a confocal microscope (Nikon A1R) from a live cell at 100x magnification. The mean lifespan of paxilin was evaluated by ImageJ.

2.4. Cell Tracking Assay and Wound Healing Assay

It is essential to evaluate the effect of MAP4K4 inhibitors on cell migration and wound healing by confirming that MAP4K4 affects FA. Breast cancer cells MDA-MB-231 (10^5 × 1 cell per millilitre after incubation overnight on a 6-well plate subsequently for 24 hours with (50nM, 500nM or 1µM) dihydrochloride PF 06260933 (TOCRIS) and 0.1% DMSO was used for the control group, live cell imaging was performed with a time-lapse microscope (Nikon Eclipse TiE), (10×magnification, every 15 minutes for 24 hours). To determine the displacement rate (micrometres per hour), the distance travelled by each cell in 24 hours was calculated.

To induce wound on a culture medium to evaluate the wound healing of breast cancer cells MDA-MB-231 (1×10^5 cells/ ml), after 90% cellular growth of culture medium in 6-cell plates, with 200 µl pipette tip, several scratches were created.

To evaluate the wound size, cells were incubated after three times washing, in DMEM media containing (1µM) dihydrochloride (TOCRIS) or 0.1 ٪ DMSO and were evaluated by Nikon Eclipse TiE microscope after 24 hours. The percentage of wounds covered was determined after 24 hours (Average initial area, t=0 h) – (Average final area, t=24 h) × 100.

2.5. Statistical Analysis

Imaging software with GraphPad Prism 5 software was used for statistical analysis of the obtained data. (San Diego, CA). One-way analysis of variance (ANOVA) and Dunnett post hoc test were performed on the data obtained from the studied groups in the current study. It should be noted that the results were obtained from at least three replications of three independent experiments (n=3). A value considered statistically significant was $P\leq0.05$.

3. Results

3.1. MAP4K4 Role in the FA Formation

To investigate the role and importance of MAP4K4 in FA dynamic, MDA-MB-231 cells were cultured with MAP4K4-wild type, MAP4K4-K54R, MAP4K4-T178D or MAP4K4-T178A. The cells were re-plated on fibronectin (20µg/ mL), fixed with paraformaldehyde, incubated with FLAG epitope-MAP4K4 and an anti-paxillin monoclonal antibody, and then stained with Dylight 549 conjugated goat anti-mouse IgG. MAP4K4-K54R, MAP4K4-T178D or MAP4K4-T178A expression stimulated FA size in MDA-MB-231 cells compared with MAP4K4 (Figure 2A). The mean size of paxillin was significantly increased with MAP4K4-K54R, MAP4K4-T178A and MAP4K4-T178D from 0.2±0.08 μm^2 to 1.8±0.05 μm^2, 1.6±0.05 μm^2 and 1.4±0.05 μm^2 (Figure 2B), while the mean paxillin number was unchanged for MAP4K4-K54R, MAP4K4-T178A and MAP4K4-T178D values changing from 42±0.9 to 40±1.4, 40±0.9 and 41±0.5 (Figure 2C). 100 paxillin-containing FA was used to assess the size and number (per experiment).

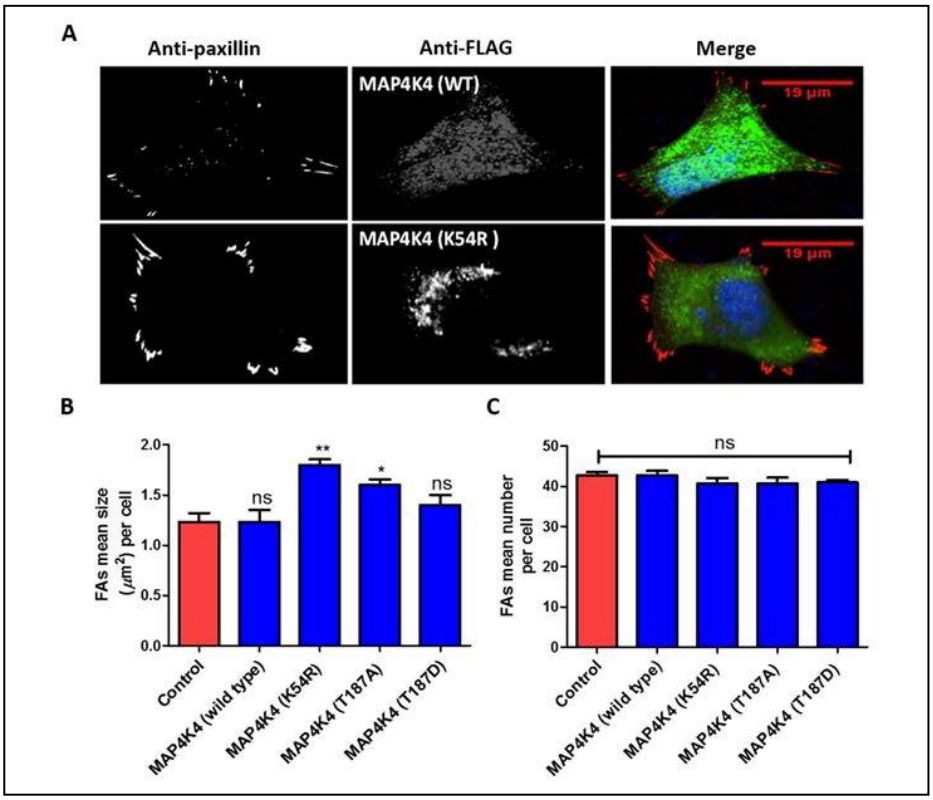

Figure 2. Effect of MAP4K4 on the FA dynamics. **(A)** Images from FA and MAP4K. **(B-C)** The data include three independent biological experiments

3.2. The Activity of *MAP4K4* in Promoting FA Dynamics

MDA-MB-231 cells were transfected with wild MAP4K4-type, MAP4K4-K54R, MAP4K4-T178D and MAP4K4-T178A and GFP-paxillin to evaluate MAP4K4 activity for FA dynamics, and then coated on ibid dishes covered with collagen (2 mg/mL). Images were recorded at 3 min intervals for 3 secs. The mean of the turnover time of paxillin was significantly increased with MAP4K4-K54R, MAP4K4-T178A, and MAP4K4-T178D compared to WT-MAP4K4 from 29.6±0.88s to 39.67±1.33s, 37.67±1.45s and 37.67±1.45s respectively (Figure 3A). Similarity (Figure 3B), compared with 0.1% DMSO, the mean of the turnover time of paxillin was significantly increased with (PF 06260933 dihydrochloride) inhibitor from 31.7±0.9 s to 44±2.3s. This result indicates that the activity of MAP4K4 is required for its stimulation of FA turnover.

3.3. MAP4K4 Role in the Cell Migration

Wound healing techniques were used over time to evaluate the migration of MDA-MB-231 cells treated with MAP4K4 PF 06260933 dihydrochloride inhibitors to investigate MAP4K4 regulates the migration of MDA-MB-231 breast cancer cells through FA dynamics. The results showed that cells treated with PF dihydrochloride displayed the speed of cell migration and covering an area 24 hours after scratching were reduced in cells treated with PF dihydrochloride compared to that of DMSO-treated cells form (22.33±0.88 µm/h^{-1}, to 16.00±1.15 µm/h^{-1} (Figure 4A). Similarly, the cover area of wound healing was reduced, compared to that of DMSO-treated cells form (67±1.2 to 20±0.57) (Figure 4B). These results suggest that MAP4K4 activity is essential for the migration of MDA-MB-231 breast cancer cells.

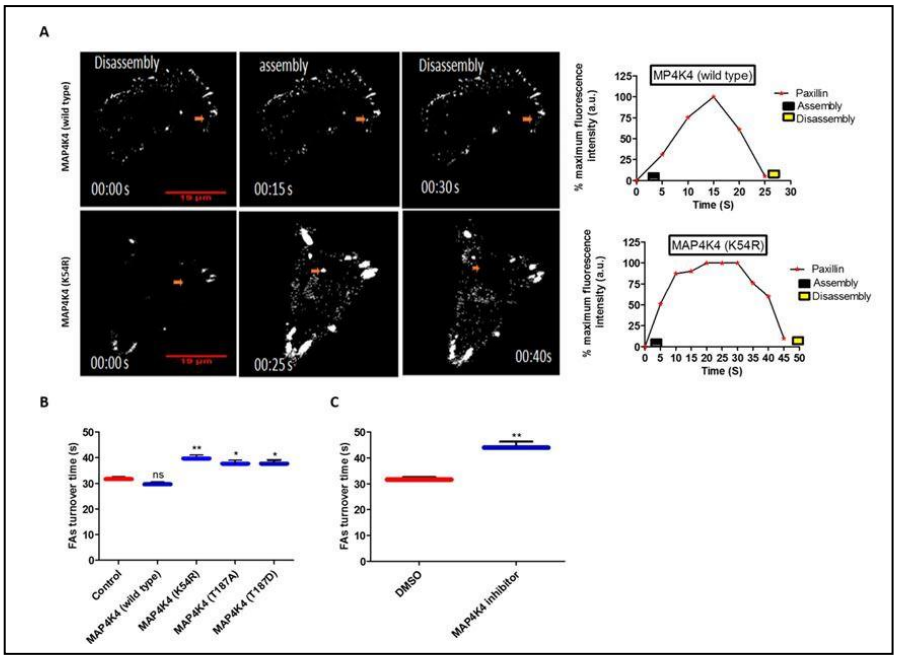

Figure 3. Effect of MAP4K4 on FA turnover duration. **(A)** Assembling and disassembling the FA White arrows represent the FA. Quantitative analysis of FA circulation time. **(B)** Comparison of the effects of MAP4K4 variants on FA dynamic

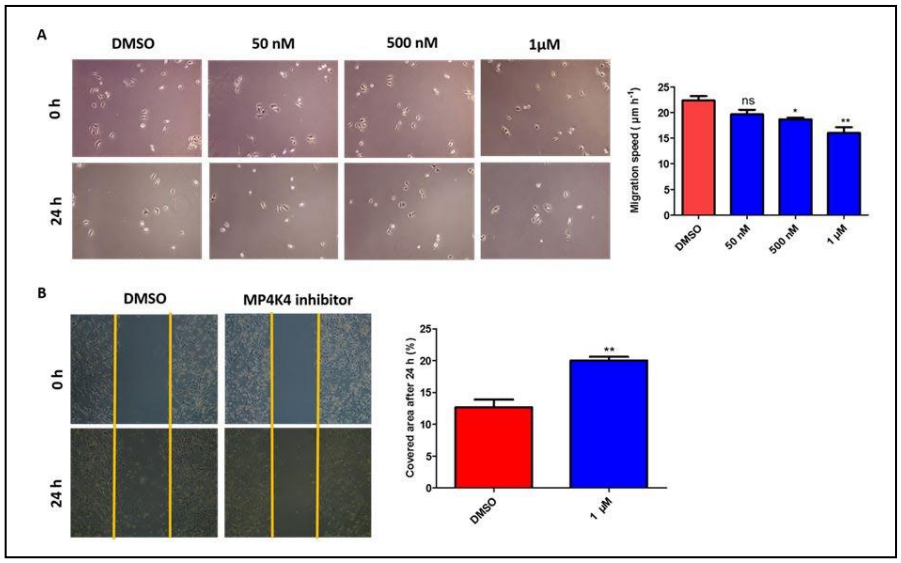

Figure 4. Effect of MAP4K4 inhibitor on cell migration. **(A)** Cellular monitoring using a time-lapse microscope over 24 h **(B)** wound healing of MDA-MB-231 cells and quantitative analysis of the entire area covered 24 hours after scratching.

4. Discussion

MAP4K4 plays a vital role in regulating several cellular processes and can affect the progress of oncogenic effects in cells (14). Although the details of its role and effects in regulating FA dynamics in cancer cell lines are not fully recognized, the present study showed that MAP4K4-K54R, MAP4K4-T178D and MAP4K4-T178A stimulated FA formation and FA circulation rate and reduced compared to WT-MAP4K4. In addition, inhibition of MAP4K4 reduced the FA circulation rate and cell migration rate. The researchers demonstrated the critical role of MAP4K4 by phosphorylating and activating various signalling pathways, such as the Arp2 / 3 complex, F-actin, and ERM and Arf6, which lead to the regulation of FA dynamics in a breast cancer cell line (15). They can subsequently activate cytoskeletal regeneration and integrin circulation, leading to increased FA circulation and cell migration (11, 16-18).

Another pathway is the function of MP4K4 as a FA regulator by binding to the microtubules of skin epidermal cells; this protein group, by myosin phosphorylation at FA sites or interaction to a β1-integrin intracellular domain, blocks the binding between integrin and talin (6).

Another ability of MAP4K4 is its role in tumour metastasis, which appears to regulate cell adhesion negatively by kinase-dependent activity. It should be noted that its role in signalling regulates the skeletal structure, and cell adhesion is also necessary. In general, MAP4K4 appears to destabilize FA, and inhibition of kinase activity leads to reduced cell migration and stabilization of the adhesion complex, which is mediated by phosphorylation of MAP4K4 molasses or other cytoskeletal proteins (6, 10).

MAP4K4 affects the invasion of cancer cells by modulating FA dynamics. The complex process of cancer metastasis is related to cell-matrix adhesion's spatial and temporal regulation. The results of this study indicate the potential role of MAP4K4 in regulating FA dynamics, as well as the role of phosphorylation of various MAP4K4 signalling pathways that are vitally involved in FA dynamics and cell migration.

Authors' Contribution

D. A., M. A. and M. C. experimented. D. A. wrote the manuscript with support from M. A. and D. A. conceived the original idea and supervised the project.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

We are very grateful to Professor Angela Clerk for giving us MAP4K4 isoforms. We would also like to thank the University of Thi-Qar for supporting us.

References

1. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science. 2003;302(5651):1704-9.
2. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673-87.
3. Alfahad D, Alharethi S, Alharbi B, Mawlood K, Dash P. PtdIns(4,5)P2 and PtdIns(3,4,5)P3 dynamics during focal adhesions assembly and disassembly in a cancer cell line. Turk J Biol. 2020;44(6):381-92.
4. Gardel ML, Schneider IC, Aratyn-Schaus Y, Waterman CM. Mechanical integration of actin and adhesion dynamics in cell migration. Annu Rev Cell Dev Biol. 2010;26:315-33.
5. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M. Turnover of focal adhesions and cancer cell migration. Int J Cell Biol. 2012;2012:310616.
6. Yue J, Xie M, Gou X, Lee P, Schneider MD, Wu X. Microtubules regulate focal adhesion dynamics through MAP4K4. Dev Cell. 2014;31(5):572-85.
7. Dourdin N, Bhatt AK, Dutt P, Greer PA, Arthur JS,

Elce JS, et al. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. J Biol Chem. 2001;276(51):48382-8.

8. Su YC, Han J, Xu S, Cobb M, Skolnik EY. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. Embo J. 1997;16(6):1279-90.
9. Yao Z, Zhou G, Wang XS, Brown A, Diener K, Gan H, et al. A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem. 1999;274(4):2118-25.
10. Vitorino P, Yeung S, Crow A, Bakke J, Smyczek T, West K, et al. MAP4K4 regulates integrin-FERM binding to control endothelial cell motility. Nature. 2015;519(7544):425-30.
11. Gao X, Gao C, Liu G, Hu J. MAP4K4: an emerging therapeutic target in cancer. Cell Biosci. 2016;6:56-.
12. Fehon RG, McClatchey AI, Bretscher A. Organizing the cell cortex: the role of ERM proteins. Nat Rev Mol Cell Biol. 2010;11(4):276-87.
13. Fuller SJ, Edmunds NS, McGuffin LJ, Hardyman MA, Cull JJ, Alharbi HO, et al. MAP4K4 expression in cardiomyocytes: multiple isoforms, multiple phosphorylations and interactions with striatins. Biochem J. 2021;478(11):2121-43.
14. Li L, He Y, Zhao M, Jiang J. Collective cell migration: Implications for wound healing and cancer invasion. Burns Trauma. 2013;1(1):21-6.
15. Xue Y, Wang X, Li Z, Gotoh N, Chapman D, Skolnik EY. Mesodermal patterning defect in mice lacking the Ste20 NCK interacting kinase (NIK). Development. 2001;128(9):1559-72.
16. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol. 2006;7(5):347-58.
17. Schwaid AG, Su C, Loos P, Wu J, Nguyen C, Stone KL, et al. MAP4K4 Is a Threonine Kinase That Phosphorylates FARP1. ACS Chem Biol. 2015;10(12):2667-71.
18. Schweitzer JK, Sedgwick AE, D'Souza-Schorey C. ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis. Semin Cell Dev Biol. 2011;22(1):39-47.