APPLICATION NOTE

A1/A1 R, AX/AX R Confocal Microscope

Membrane dynamics of recycling endosomes detected by confocal microscopy

Membrane deformation, which elongates the recycling endosome into a tubular structure, plays an important role when endocytosed functional molecules such as receptors and adhesion molecules are recycled back to the cell membrane surface. The recycling of functional molecules contributes to various cell functions such as cell proliferation, migration, and differentiation. Therefore, dysregulation of the membrane dynamics of recycling endosomes could cause abnormal tissue/organ formation and cancer metastasis. Although many molecules are known to be involved in the formation of tubular recycling endosomes, the detailed molecular mechanisms remain unclear.

Dr. Ayuko Sakane and Dr. Takuya Sasaki of the Department of Biochemistry, Graduate School of Medicine, Tokushima University, found a new mechanism in which recycling endosomes, one of the organelles involved in intracellular vesicular trafficking, exhibit dynamic membrane deformation.

Confocal microscopes can acquire not only ordinary confocal images, but also high-resolution images in various applications such as 3D construction, time-lapse imaging, and FRAP (fluorescence recovery after photobleaching). In 2021, Dr. Sakane et al. captured images of the membrane deformation of recycling endosomes and clarified the molecular mechanism thereof. This application note introduces the findings reported by Dr. Sakane et al., focusing on the contribution of confocal microscopy.

Keywords: confocal microscopy, recycling endosome, membrane deformation, liquid-liquid phase separation, FRAP analysis

Overview

Recycling endosomes form long tubular structures composed of lipid bilayer membrane, and play an important role when various endocytosed functional molecules such as receptors and adhesion molecules are recycled back to the cell membrane surface.

Rab family small G proteins are known to be involved in the regulation of intracellular vesicular trafficking. Each Rab protein is exchanged between a GTP-bound active form and GDP-bound inactive form. Active Rab proteins have specific interacting effector proteins, and perform their functions via Rab-effector protein complexes. Therefore, to understand the cellular function of a Rab protein, it is first necessary to identify its effector protein.

In previous work in Dr. Sasaki's Laboratory, JRAB was identified as an effector protein of Rab8A and Rab13. In this study, Dr. Sakane et al. focused on the JRAB-Rab8A complex, and attempted to elucidate the regulatory mechanism of the membrane dynamics of recycling endosomes.

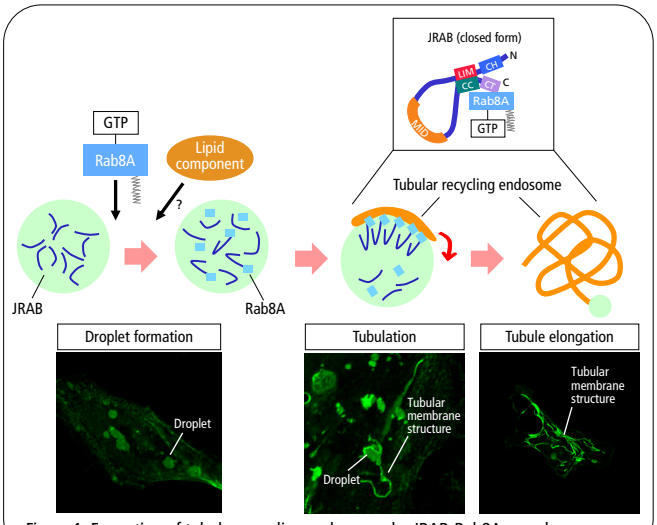

Figure 1. Formation of tubular recycling endosomes by JRAB-Rab8A complex

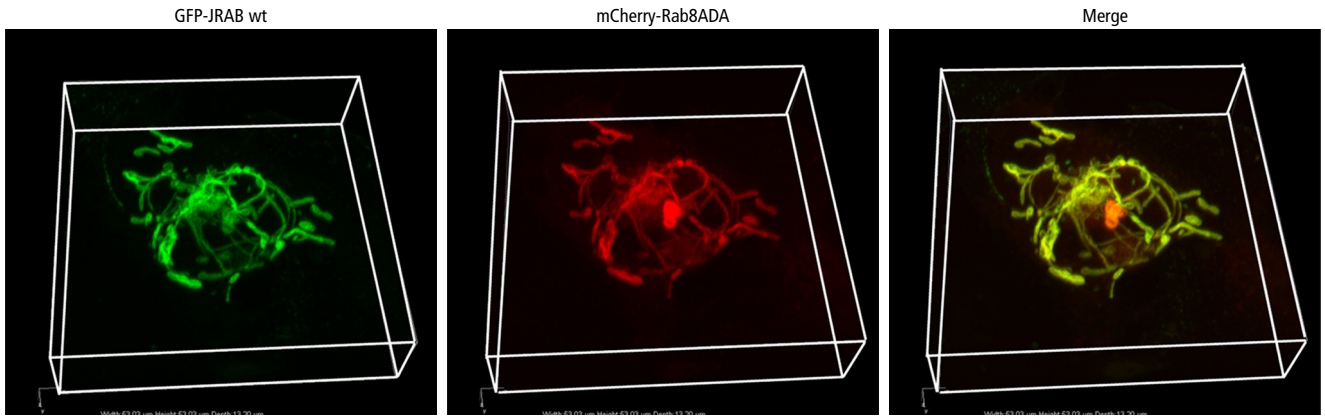

Figure 2. 3D image of HeLa cells expressing GFP-JRAB and mCherry-Rab8ADA
Objective: CFI Plan Apochromat VC 60XC WI, NA 1.2

Results

Confocal 3D imaging revealed that JRAB co-localized with Rab8ADA (an active mutant) in tubular structures of recycling endosomes in HeLa cells expressing GFP-JRAB and mCherry-Rab8ADA (Fig. 2).

Next, it was examined how JRAB and Rab8A form tubular structures of recycling endosomes. In HeLa cells expressing GFP-JRAB and HA-Rab8ADA, droplet-like structures (arrows) were observed as pre-tubular structures. Long tubular structures of recycling endosomes were derived from the fused droplet-like structures (Fig. 3).

Furthermore, the possibility was raised that JRAB and Rab8A undergo liquid-liquid phase separation (LLPS) and the pre-tubular structures are liquid-like droplets. To verify this possibility, the mobility of proteins inside and around droplets, which is a characteristic of liquid-like droplets observed in LLPS, was examined. An FRAP assay was performed by confocal microscopy to monitor the fluorescence intensity of GFP-JRAB, and a GFP signal with a half-time of 24 seconds was recovered after bleaching (Fig. 4). A comparison of this result with the exchange rate of molecules in various intracellular condensates indicated that the JRAB droplets are not immobile protein aggregates.

Also, it was investigated whether JRAB and Rab8A formed LLPS droplets *in vitro*. The result was that GFP-JRAB and mCherry-Rab8ADA underwent LLPS and formed droplets upon addition of PEG (polyethylene glycol), a water-soluble polymer (Fig. 5).

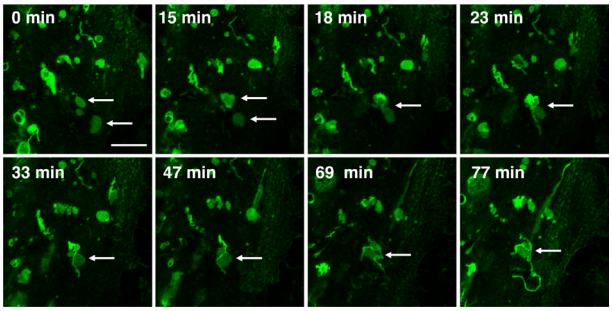

Figure 3. Time-lapse imaging of HeLa cells expressing GFP-JRAB and HA-Rab8ADA
Scale bar: 10 µm
Objective: CFI Plan Apochromat VC 60XC WI, NA 1.2

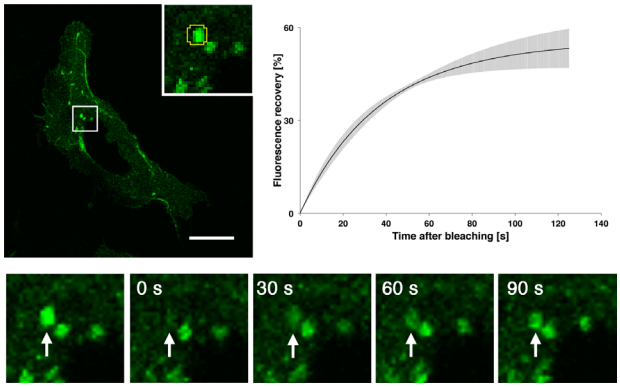

Figure 4. FRAP analysis of HeLa cells expressing GFP-JRAB
Scale bar: 25 µm
Objective: CFI Plan Apochromat Lambda 40XC, NA 0.95

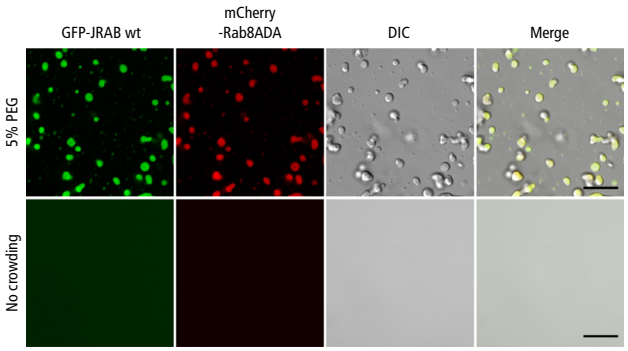

Figure 5. *In vitro* droplet reconstitution experiments using recombinant proteins GFP-JRAB and mCherry-Rab8ADA
Scale bar: 10 µm
Objective: CFI Plan Apochromat VC 60XC WI, NA 1.2

Summary

Using confocal microscopy, it was revealed that Rab8A localizes to recycling endosomes via its lipid-modified C-terminal region, and recruits JRAB there. It was shown that the recruited JRAB becomes a closed form through binding with Rab8A, resulting in the membrane deformation of recycling endosomes and formation of tubular structures. In addition, it was suggested through multifaceted approaches such as live imaging, FRAP, and *in vitro* droplet reconstitution experiments that the origin of the tubular structure of the recycling endosome that was formed by the JRAB-Rab8A complex is the condensed droplet formed in the cell by phase separation of JRAB and Rab8A. Recycling of receptors and adhesion molecules contributes to various cellular functions such as cell proliferation, migration, and differentiation, and its dysregulation is involved in abnormal tissue/organ formation and cancer metastasis.

Therefore, the elucidation of a part of the regulatory mechanism of the membrane dynamics of recycling endosomes would provide a better understanding of developmental abnormalities and cancer metastasis mechanisms in the future.

Acknowledgements

We would like to express our sincere gratitude to Dr. Ayuko Sakane and Dr. Takuya Sasaki of the Department of Biochemistry, Graduate School of Medicine, Tokushima University for their full cooperation in the production of this application note.

Reference

"JRAB/MICAL-L2 undergoes liquid–liquid phase separation to form tubular recycling endosomes"

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Communications Biology, Volume 4, Article number: 551 (2021)

https://doi.org/10.1038/s42003-021-02080-7

Product information

AX/AX R Confocal Microscope

Supports high-speed, high-resolution, large field-of-view confocal imaging, with reduced phototoxicity to living cells and photobleaching.

- High speed: Up to 720 fps (resonant at 2048 x 16 pixels)
- High resolution: Up to 8K (galvano)/2K (resonant)
- High throughput: Ultra-wide FOV of 25 mm