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석사학위 논문

# Complementary Functions of *Dictyostelium* Rap1 Deactivating Proteins in Cell Migration and Development

조 선 대 학 교 대 학 원

생 명 과 학 과

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세포이동과 발생분화에서 *Dictyostelium* Rap1 비활성화 단백질들의 상호 보완적 기능

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지도교수 전택중

이 논문을 이학석사학위 신청 논문으로
제출함

2016년 4월

조 선 대 학 교 대 학 원

생 명 과 학 과

이 아 라

# 이아라의 석사 학위논문을 인준함

위원장 조선대학교 부교수 이준식 (인)

위 원 조선대학교 부교수 조광원 (인)

위 원 조선대학교 부교수 전택중 (인)

2016년 5월

조 선 대 학 교 대 학 원

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# ABBREVIATIONS

| | |
|---|---|
| cAMP | cyclic adenosine monophosphate |
| cAR | cyclic AMP receptors |
| DAPI | 4', 6'-diamidine-2'-phenylidole dihydrochloride |
| GAP | GTPase activating proteins |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green fluorecent protein |
| GPCR | G-protein coupled receptor |
| GTP | Guanosine triphosphate |
| RapGAP | Rap1-specific GTPase-activating proteins |

# Abstracts

## Complementary Functions of *Dictyostelium* Rap1 Deactivating Proteins in Cell Migration and Development

LEE, ARA

Advisor: Associate Prof. Taeck Joong Jeon, Ph.D.

Department of Life Science,

Graduate School of Chosun University

The small GTPase Rap1 is an important regulator in cell adhesion and cell migration. Recently, it has been reported that spatial and temporal regulation of Rap1 activity by Rap1-specific GTPase-Activating Proteins (RapGAPs) is required for development and cell migration in *Dictyostelium*. RapGAP1, a Rap1-specific GAP protein, is involved in spatiotemporal regulation of cell adhesion at the front of chemotaxing cells. RapGAP3 is associated with

morphogenesis and development, and RapGAP9 is involved in cell adhesion and cytokinesis. *rapGAP1* knockout cells had a higher cell substratum adhesion than wild-type, and *rapGAP3* knockout cells had defects in the late mound stage, delaying fruiting body formation. Cells lacking RapGAP9 showed flatten and spread morphology compared to wild-type cells, and increased cell substratum adhesion. In this study, I investigated overlapping/specific functions of RapGAP1, RapGAP3, and RapGAP9 in morphogenesis, cytokinesis, development, and adhesion by analyzing phenotypes of *rapGAPs* null cells and *rapGAPs* null cells expressing each RapGAP or GAP domain. First, I analyzed the morphogenesis and cytokinesis defects of *rapGAP9* null cells. The cell size and cytokinesis defects of *rapGAP9* null cells were partially complemented by overexpressing RapGAP1 or RapGAP3, suggesting that functions of RapGAP1, RapGAP3, and RapGAP9 are partially overlapped in the processes of morphogenesis and cytokinesis. Next, I examined the complementation of RapGAP proteins in developmental processes. The developmental defects of *rapGAP3* null cells were partially complemented by

RapGAP9, but not by RapGAP1. The adhesion defects of *rapGAP1* null cells were not complemented by overexpressing either RapGAP3 or RapGAP9. On the other hand, interestingly the adhesion defects of *rapGAP9* null cells were partially complemented by RapGAP1 or RapGAP3. These results indicate that the functions of RapGAP1, RapGAP3, and RapGAP9 are overlapped and might be redundant in the processes of morphogenesis and cytokinesis. However, in the developmental process, only RapGAP3 and RapGAP9 appear to have overlapped roles while only RapGAP1 and RapGAP9 have partially complementary functions in cell adhesion. The present study investigated the functional interactions among RapGAP proteins, RapGAP1, RapGAP3, and RapGAP9 and determined overlapping/specific functions of RapGAP proteins in several biological processes including morphogenesis, cytokinesis, development, and cell adhesion. These results are expected to contribute for further understanding Rap1 signaling pathway and Rap1-mediated various cellular biological processes.

# 국문초록

## 세포이동과 발생분화에서 *Dictyostelium* Rap1 비활성화 단백질들의 상호 보완적 기능

Small GTPase Rap1은 세포 부착과 세포 이동의 중요한 조절 단백질로 알려져 있다. 최근의 연구에 의하면, *Dictyostelium* 세포에서 발생 과정과 세포 이동 시, Rap1 비활성화 단백질들 (RapGAPs)에 의한 Rap1 활성의 시간적 공간적 조절이 매우 중요한 것으로 밝혀지고 있다. RapGAP1은 Rap1-특이적 GAP 단백질로써 주화성 이동을 하는 세포의 앞쪽에서 세포의 부착을 조절하는 것으로 알려져 있으며, RapGAP3는 세포의 형태 형성과 발생 과정에서 중요한 역할을 하는 것으로 밝혀지고 있다. 최근에 동정된 RapGAP9은 세포 부착과 세포질 분열에 연관되어 있는 것으로 알려져 있다. *Dictyostelium* 세포의 성장 단계에서, RapGAP1이 없는 세포는 야생형의 세포에 비해 세포-바닥간의 부착이 증가하며, RapGAP3가 없는 세포는 야생형의 세포에 비하여 late mound stage에 결함이 생겨 과실체의 형성이 지연되며, RapGAP9이 없는 세포는 야생형의 세포에 비해 더 납작해지고 편평해져 세포의 크기가 증가하고 세포-

바닥 간의 부착이 증가한다. 또한 세포질 분열에서 RapGAP9이 없는 세포는 야생형의 세포에 비해 다핵을 가지는 세포의 비율이 증가한다. 이를 바탕으로 하여, 본 연구에서는 RapGAP1, RapGAP3, RapGAP9 각각에 대한 knock-out 세포주와 과발현 세포주, 및 절편 단백질 발현 세포주를 이용하여 세포의 형태 형성, 세포질 분열, 발생, 세포 부착에서 각 단백질들의 상호 보완적기능과 특이적 기능들이 무엇인지를 밝히고자 하였다. 세포 형태 형성에서 *rapGAP9*이 없는 세포에서 세포의 크기가 증가하는 결함은 RapGAPs의 과발현에 의해서 부분적으로 보완되며, 각 RapGAP의 GAP 절편 단백질의 발현에 의해서도 부분적으로 보완되었다. 세포질 분열에서 *rapGAP9*이 없는 세포에서의 다핵을 가지는 결함이 RapGAP의 발현에 의해서 다핵을 가지는 비율이 감소하였으며 GAP 절편 단백질의 발현에 의해서도 부분적으로 감소하였다. 세포 발생에서, *rapGAP3*가 없는 세포에서의 발달의 지연이 나타나는 결함은 RapGAP9의 과발현에 의해서 부분적으로 보완되었으나 RapGAP1의 과발현에 의해서는 보완되지 않았다. 또한 RapGAP9의 GAP 절편 단백질의 과발현에 의해서는 부분적으로 보완되었으나 각 RapGAP1, RapGAP3의 GAP 절편 단백질의 과발현에 의해서는 보완되지 않았다. 세포 부착에서 *rapGAP1*이 없는 세포에서의 세포-바닥 간 부착이 증가하는 결함은 RapGAP3와 RapGAP9의 과발현에 의해서 세포-바닥 간의 부착성이 부분적으로 감소되었으나 보완되지

않았으며 각 RapGAP의 GAP 절편 단백질의 과발현에 의해서도 보완되지 않았다. 또한 *rapGAP9*이 없는 세포에서의 세포-바닥 간의 부착이 증가하는 결함은 RapGAPs의 과발현에 의해서 부분적으로 보완되며 각 RapGAP의 GAP 절편 단백질의 과발현에 의해서 부분적으로 보완되었다. 이는 세포 형태 형성과 세포질 분열에서 RapGAP 단백질 간의 기능이 공유되며 서로 보완이 가능하고 발생 과정에서는 RapGAP3와 RapGAP9의 기능이 서로 부분적으로 공유되며, 세포 부착에서는 RapGAP1과 RapGAP9의 기능이 부분적으로 공유된다는 것을 나타내 주는 결과이다. 본 연구에서는 Rap1-특이 비활성 단백질들 간의 기능적 상호보완성과 고유성에 대한 연구를 하였으며, 본 연구 결과는 Rap1 신호 전달 경로에 의해 매개되는 다양한 세포 생명 현상을 이해하는데 크게 기여할 것으로 기대된다.

# I. Introduction

Chemotaxis or directional movement toward a chemical compound is an essential property of many cells. For example, chemotaxis is critical for the recruitment of leukocytes to the sites of infection, tracking of lympocytes in the human body, and neuronal patterning in the developments of nervous system. Research on eukaryotic chemotaxis has progressed substantially, mainly through the study of the amoeba *Dictyostelium discoideum* as a model system (Kimmel and Parent, 2003; Williams et al., 2006). Multicellular developmental process of *Dictyostelium* is initiated by starvation, during which cAMP pulse is generated in cells and transduced towards the periphery. cAMP acts as a chemoattractant and induces chemotaxis, resulting in aggregation and mound formation. This eventually culminates in multicellular fruiting body formation (Dormann and Weijer, 2006). As the mechanism of chemotaxis is essentially identical in all eukarytes, *Dictyostelium* offers a genetically tractable model for studying chemotaxis. The first step in chemotaxis is the binding of chemoattractants to the cell surface G-protein coupled receptors (GPCRs). In *Dictyostelium* four cAMP receptors (cAR) have been identified. cAR1 has a high affinity for cAMP and is essential for signal transduction during early development and chemotaxis, whereas cAR2-4 have a relative low affinity for cAMP and are important for multicellular devemopment (Loomis, 2014).

Cell migration is involved in many biological and pathological processes, including embryonic development, wound healing, inflammatory responses, and tumor cell invasion and metastasis (Lee and Jeon, 2012). Also, cell

migration is an integrated process that requires the continuous, coordinated formation and disassembly of cell attachment. The cell migration mechanism incudes extension of an F-actin mediated protrusion, formation of stable attachment near the leading edge, translocation of the cell body forward, release of the attachment, and retraction of the cell's posterior drived by myosin II-mediated contraction (Lee and Jeon, 2012).

Human oncogene Ras proteins have been the subject of intensive research. The *Dictyostelium* Ras GTPase subfamily is comprised of 15 proteins; 11 Ras, 3 Rap, and one Rheb related protein (Kortholt and van Haastert, 2008). Ras proteins are active when GTP is bound and become inactive by hydrolyzing this GTP to GDP. Two families of proteins regulate Ras activity by controlling the bound nucleotide. Guanine nucleotide exchange factors (GEFs) activate Ras by allowing GDP to dissociate and be replaced by GTP. On the other hand, GTPase-activating proteins (GAPs) inhibit Ras activity by binding to the active form and stimulating the hydrolysis of GTP to GDP. Rap1 is the closest homolog of the small GTPase Ras and, like Ras, cycles between an inactive GDP-bound and an active GTP-bound conformation (Bos and Zwartkruis, 1999). Also, Rap1 plays important roles in the regulation of cell adhesion during chemotaxis. Recent reports have demonstrated that Rap1 is rapidly and transiently activated in response to chemoattractant stimulation (Jeon et al., 2007b). Cells expressing constitutively active Rap1 have high adhesion and unable to effectively control myosin II assembly and disassembly. Rap1 regulates cell adhesion and cell polarity by modulating myosin II assembly and disassembly via Phg2. Rap1 is also involved in F-actin polymerization through Rac signaling pathway (Lee and Jeon, 2012). RapGAP1 was recently identified

as a specific GAP protein for Rap1 and is involved in the temporal and spatial regulation of Rap1 activity in the anterior of chemotaxing cells to control cell substratum adhesion and Myosin II assembly during chemotaxis (Jeon et al., 2007a). RapGAP3 regulates the levels of Rap1 activation during morphogenesis and mediates the proper sorting of prestalk and prespore cells within the multicellular aggregate by controlling cell–cell adhesion and cell migration (Jeon et al., 2009). RapGAP9 was identified as a putative specific GAP protein for Rap1 and is involved in the morphogenesis and development (Mun et al., 2014).

To further examine the functional interactions among RapGAP proteins, I performed complementary experiments. In this study, I delineate distinct overlapping functions of RapGAPs in morphogenesis, cytokinesis, development, and adhesion in *Dictyostelium* cells.

# II. Materials & Methods

## II-1. Cell culture and Strains

*Dictyostelium* strains were grown in HL5 axenic media at 22 ℃. For expression of full length RapGAPs or GAP domain proteins in *rapGAP* null cells, the various RapGAP or GAP domain sequences were amplified by PCR and cloned into *EcoR* I-*Xho* I site of an pEXP-4(+) vector. *rapGAP* knock-out cells expressing GFP-tagged RapGAPs proteins and GFP-tagged GAP domain of RapGAPs are made by electroporesis. Randomly selected clones were screened for a 20 ㎕/ml G418. The GFP-tagged transformants were maintained in 20 ㎍/ml G418.

## II-2. Cell adhesion

Log-phase growing cells on the plates were washed with Na/K phosphate buffer. And then resuspended at a density of 2 x $10^6$ cells/ml. The amount of 4 x $10^5$ cells in 200 ㎕ were placed on the 6 well culture dish for over night. The cells were photographed and counted (total cell number). To quantify attatched cell number, plates were then shaken at 180 rpm. for 1h, after that the medium was removed. The number of attached cells in plates were counted (attached cell). Calculate the percentage of the total cell and attached cell.

### II-3. DAPI staining

To determine nuclei number, cells were placed on the cell culture dish for 30 minutes. And then cells were washed with PBS. Staining with 0.5% Hoechest dye in PBS. Cell image of random fields if view were captured by using NIS-elements software (Nikon).

### II-5. Development assay

The Development assay was performed as described previously (Jeon et al., 2009). Expoteintially growing cells were harvested and washed twice with 12mM Na/K phosphate buffer (pH 6.1) and plated on Na/K phosphate agar plates at a density of 3.5 x $10^7$ cells/$cm^2$. The developmental morphology of the cells was examined by photographing the developing cells with a phase-contrast microscope.

# III. Results

## III-1. Computer based analysis of RapGAP proteins

There are nine putative Rap1GAP proteins in *Dictyostelium* genome sequence database (Table 1). Among them, RapGAP1, RapGAPB, RapGAP3, and RapGAP9 have been characterized and their functions have been studied in various biological processes (Table 1). *Dictyostelium* RapGAP1 is composed of 1055 amino acids and posses a F41 domain at the N-terminal region and a GAP domain at the C-terminal region. RapGAP3 has three PH domains at the N-terminal region and a GAP domain at the C-terminal region. RapGAP9 is a relatively small protein composed of 366 amino acids and contains a GAP domain at the C-terminal region (Fig. 1A). Phylogenetic analysis of *Dictyostelium* RapGAP proteins and human RapGAP proteins show a high homology between RapGAP3 and RapGAP9 (Fig. 1B).

**Table 1. Overview of *Dictyostelium* RapGAP proteins**

| Protein | Protein size | Location | Localization | Effect of Disruption | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Morphology | Adhesion | F-actin | Development | Cytokinesis | Chemotaxis |
| **RapGAP1** | 1055 a.a | Chromosome 2 | Membrane | No phenotype | Increased cell-substrate adhesion | Increased basal F-actin level | No phenotype | No phenotype | Move slightly slower than wild type, but not severe |
| **RapGAPB** | 418 a.a | Chromosome 3 | . | . | Increased cell-substrate adhesion | Abnormal F-actin organization | Abnormal tip mound structure | - | No phenotype |
| **RapGAP3** | 1167 a.a | Chromosome 2 | Membrane | No phenotype | No phenotype | - | Loose aggregate, form ring like structure, Delay normal fruiting body Formation | No phenotype | No phenotype |
| **RapGAP4** | 1031 a.a | Chromosome 6 | - | Long cell length | Decreased cell substrate adhesion | - | No phenotype | - | - |
| **RapGAP5** | 855 a.a | Chromosome 2 | Membrane | Increased cell size | Increased cell-substrate adhesion | - | Decreased spore size, Delay fruiting body formation | Multinucleated | - |
| **RapGAP6** | 1049 a.a | Chromosome 4 | Cytosol | Increased cell size | - | - | No phenotype | - | - |
| **RapGAP9** | 366 a.a | Chromosome 4 | Membrane | Increased cell size | Increased cell-substrate adhesion | Increased basal F-actin level | Decreased spore size, Delay fruiting body formation | Abnormal cytokinesis, Multinucleated | Decreased chemotaxis to cAMP |

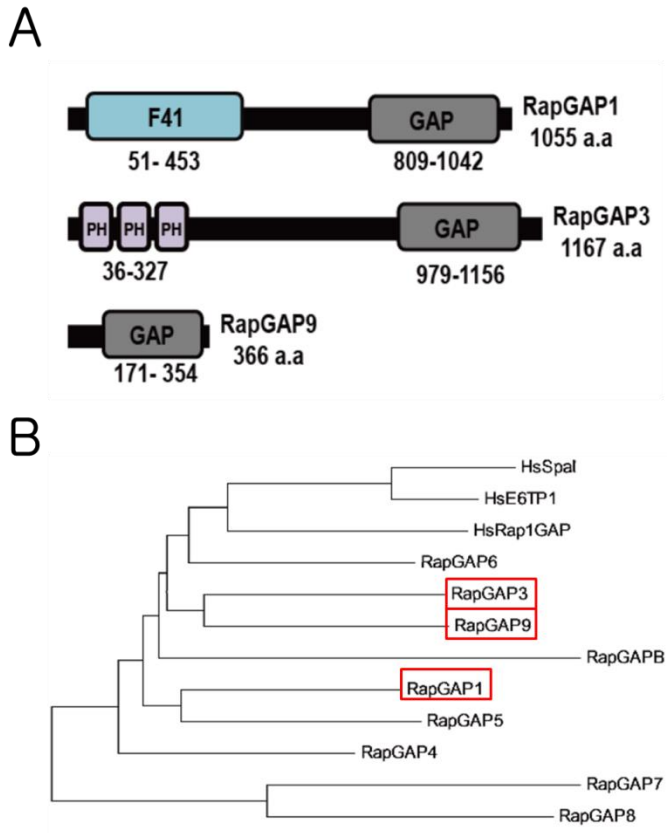


Fig.1. Domain structure and phylogenetic tree of RapGAP proteins.

(A) Domain structure of RapGAP1, RapGAP3, and RapGAP9. (B) Phylogenetic tree analysis of the RapGAPs from *Dictyostelium* and other organisms. HsSpa1, *H. sapiens* Spa1 (O60618); HsE6TP1, *H. sapiens* (Q9UNU4); HsRap1GAP, *H. sapiens* (P47736); DRapGAP1, *Dictyostelium discoideum* (dictyBaseID: DDB0233726); DRapGAPB, *Dictyostelium discoideum* (dictyBaseID: DDB0233728); DRapGAP3, *Dictyostelium discoideum* (dictyBaseID: DDB0229869); DRapGAP9, *Dictyostelium discoideum* (dictybaseID:DDB0233724); and DRapGAP6, *Dictyostelium discoideum* (dictybaseID:DDB0233725). These sequences are available at www.dictybase.org.

## III-2. Morphology

### 1) RapGAP9 is involved in the regulation of cell morphology

To determine the possible roles of *rapGAP*s in morphogenesis, I examined and compared cellular morphologies of wild-type cells and *rapGAP* null cells including, *rapGAP1*, *rapGAP3*, and *rapGAP9* null cells (Fig. 2A). Some of *rapGAP9* null cells were highly spread and flatten, compared to wild-type, *rapGAP1* null, and *rapGAP3* null cells. Measurement of cell areas using NIS software show that *rapGAP9* null cells were 2.5-fold larger than other cells including wild-type, *rapGAP1* null cells, and *rapGAP9* null cells (Fig. 2B). Histogram analysis of cell areas shows that majority of *rapGAP9* null cells have approximately 200 $\mu m^2$ of cell area, compared to approximately 100 $\mu m^2$ in other cells. These results suggest that RapGAP9 plays an important in morphogenesis.

### 2) RapGAP proteins have partially overlapped functions in morphogenesis

To determine if other RapGAPs could complement morphological defects of *rapGAP9* null cells, I prepared *rapGAP9* null cells overexpressing full-length RapGAP proteins or GAP-domain fragments. Compared to *rapGAP9* null cells, *rapGAP9* null cells expressing full-length RapGAP proteins were less flat and spread out. Although sizes of *rapGAP9* null cells expressing RapGAP proteins were smaller than that of *rapGAP9* null cells, they were approximately 1.5-fold larger than wild-type (Fig. 2). Since the morphological defects of *rapGAP9* null cells were complemented by overexpressing any RapGAP proteins, I

postulated a possibility that the GAP domain alone might show the complementory effects on *rapGAP9* null cells in morphogensis. As expected, the morphological defects of *rapGAP9* null cells were partially recovered by overexpressing GAP-domain fragments from RapGAP proteins (Fig. 3). These results suggest that RapGAP1, RapGAP3, and RapGAP9 have overlapped and redundant functions in morphogenesis. In addition, our results indicate that the GAP domain alone from RapGAP proteins tested here are sufficient for recovery of morphological defects of *rapGAP9* null cells.

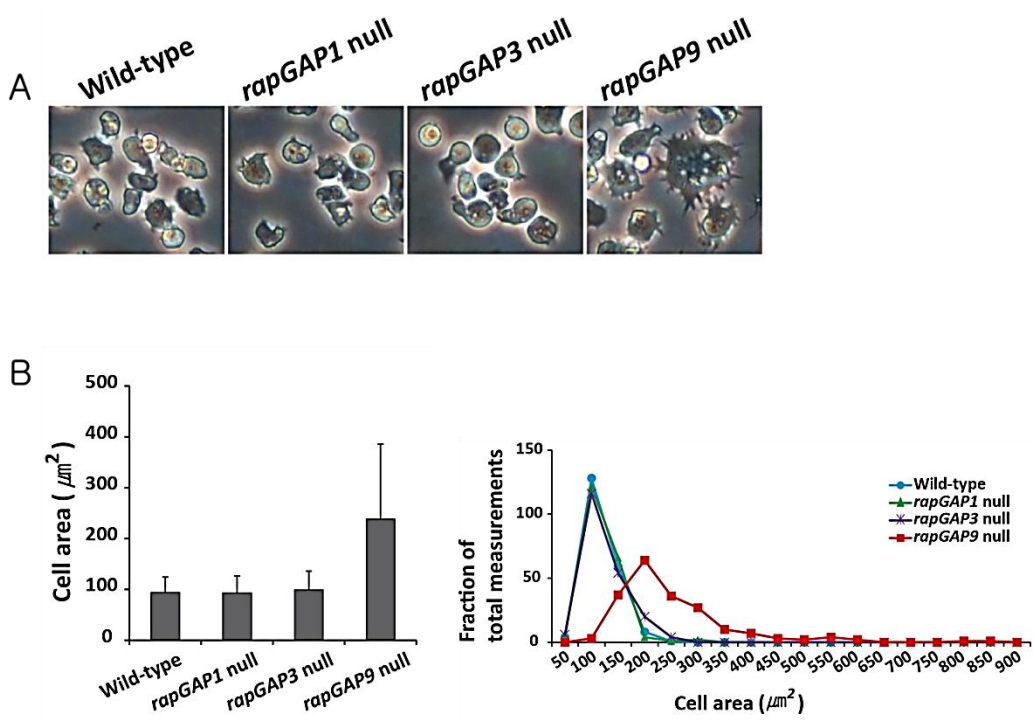


Fig.2. Cell spreading, and cell area of *rapGAPs* null cells.

(A) Morphology of *rapGAP* null cells. (B) Measurement of cell areas of *rapGAP* null cells using NIS software (left) and histogram of cell area of the null cells (right).

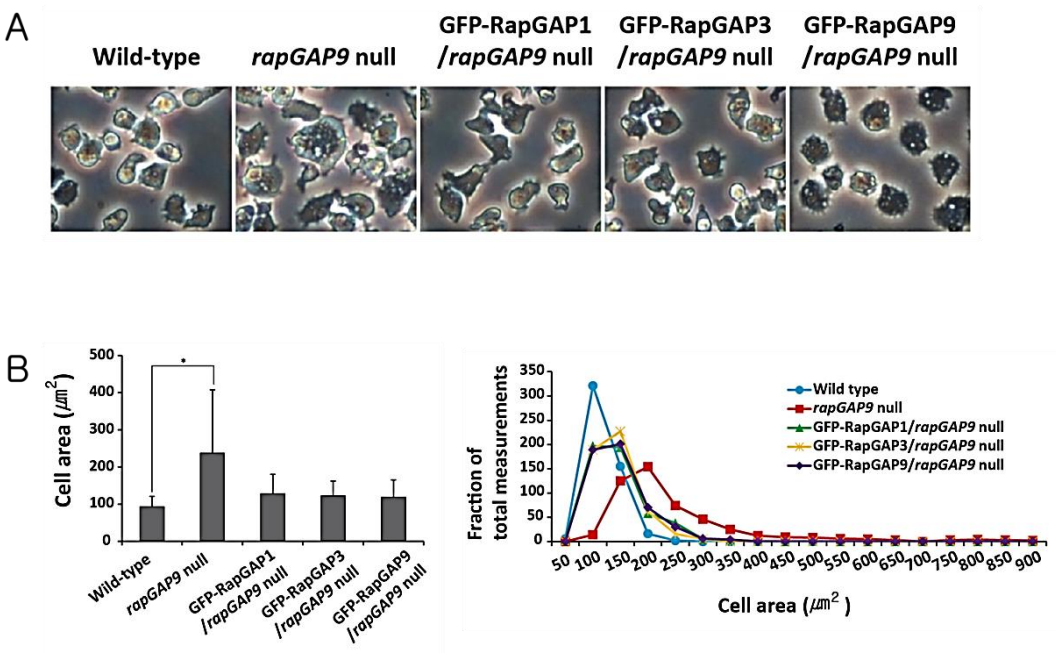


Fig.3. Cell spreading, cell area of *rapGAP9* null cells and *rapGAP9* null cells expressing RapGAPs.

(A) Morphology of *rapGAP9* null cells expressing RapGAP proteins. (B) Measurement of cell areas of *rapGAP9* null cells expressing RapGAP proteins using NIS software (left) and histogram of cell area of the cells (right). Error bars are SE values. Statistically different from control at $^{*}p<0.05$ by the student's t-test.

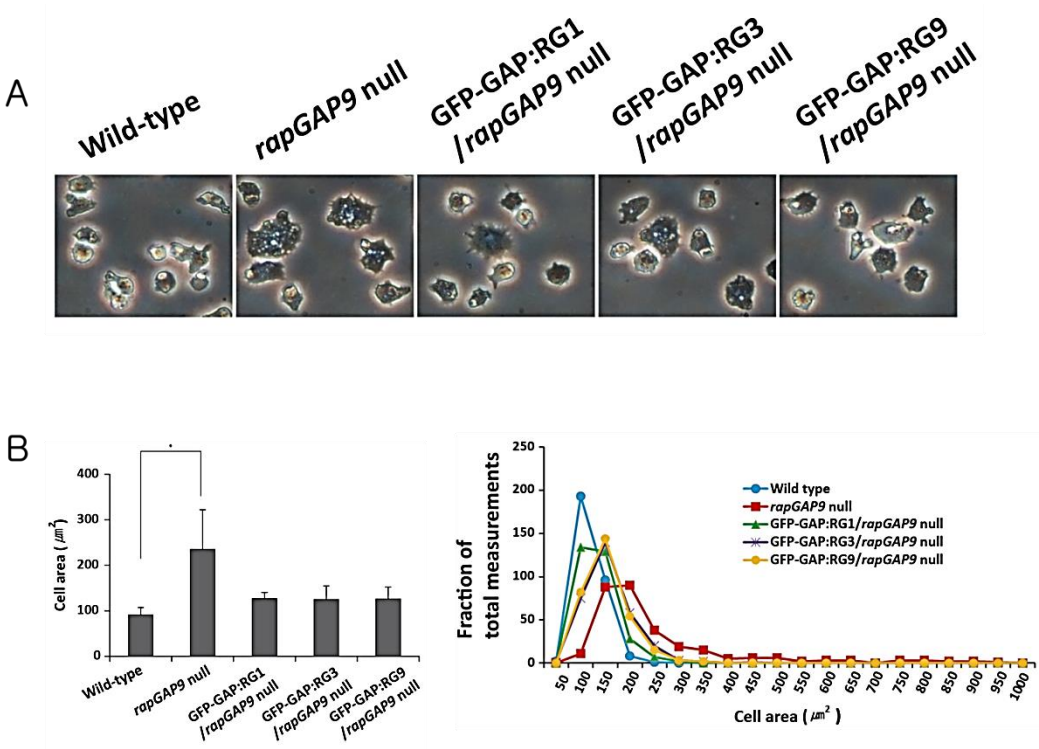


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Fig.4. Cell spreading, cell area of *rapGAP9* null cells and *rapGAP9* null cells expressing GAP domains of RapGAPs.

(A) Morphology of *rapGAP9* null cells expressing GAP-domain fragments from RapGAP proteins. (B) Measurement of cell areas. Error bars represent SE. Statistically different from control at $^{*}p<0.05$ by the student's t-test.

## III-3. Cytokinesis

### 1) RapGAP9 is involved in the regulation of cytokinesis

To investigate the possible roles of *rapGAP*s in cell cytokinesis, I counted and compared the number of nuclei in wild-type, *rapGAP1* null, *rapGAP3* null, and *rapGAP9* null cells (Fig. 5). *rapGAP9* null cells were highly multinucleated, compared to wild-type, *rapGAP1* null, and *rapGAP3* null cells, indicating that *rapGAP9* null cells have cytokinesis defects and that RapGAP9 plays an important role in cytokinesis.

### 2) RapGAP proteins have overlapped functions in cytokinesis

To determine if other RapGAP proteins could complement cytokinesis defects of *rapGAP9* null cells, I compared the number of nuclei in wild-type, *rapGAP9* null cells, and *rapGAP9* null cells overexpressing RapGAP proteins or GAP-domain fragments of RapGAP proteins. Compared to *rapGAP9* null cells, *rapGAP9* null cells expressing RapGAPs or GAP-domain fragments partially rescued cytokinesis defects of the *rapGAP9* null cells and most of the overexpressing cells had similar number of nuclei to wild-type cells (Fig. 6 and 7). These results indicate that cytokinesis defects of *rapGAP9* null cells were partially complemented by overexpression of RapGAPs and suggest that the functions of RapGAP1, RapGAP3, and RapGAP9 in cytokinesis are in part overlapped.

### 3) RapGAP9 plays an important role in cytokinesis type A and B

Cytokinesis of *Dictyostelium* is mediated by two dominant pathways, ‘cytokinesis A’ and ‘cytokinesis B'. ‘Cytokinesis A’ is a myosin II dependent pathway and is processed by active constriction of equatorial cleavage furrow. The mutant cells which have a defect in cytokinesis type A usually show normal growth on plate but multinucleated in suspension culture (Adachi, 2001). In contrast, ‘cytokinesis B’ is a myosin II independent pathway and uses traction force for segregation of daughter cells during cell division (Fig. 8A). The mutant cells with defects in cytokinesis type B exhibit abnormal growth and are multinucleated in both plate-attached culture and suspension culture. Examination of nuclei in cells by Hoechest-dye staining revealed that *rapGAP9* null cells had multi-nuclei and defects in cytokinesis. To determine whether RapGAP9 is involved in cytokinesis through either cytokinesis A or B, I examined the cytokinesis defects of *rapGAP9* null cells which were grown on plates or were cultured in suspension liquid. (Fig. 8B and C). Majority of wild-type cells had one or two nuclei on plate culture or suspension culture. In contrast, *rapGAP9* null cells were multinucleated on plate culture and the proportion of multinucleated cells were increased in suspension culture. These results suggest that RapGAP9 is involved in the regulation of cytokinesis and plays an important role in both cytokinesis A and B. To further understand cytokinesis process in *rapGAP9* null cells, cell division process of *rapGAP9* null cells was monitored. Wild-type cells became rounded in the begining of cytokinesis followed by formation of cleavage furrow and invagination, which resulted in two identical daughter cells. In

contrast, *rapGAP9* null cells showed regression after formation of multiple cleavage furrows, resulting in a failure of cell division, even though some *rapGAP9* null cells showed normal cell division as wild-type cells. The *rapGAP9* null cells became rounded a few hours later, followed by formation of multiple cleavage furrows and division into three asymmetrical daughter cells (Fig. 8D). The percentages of completed cell division of the cells were quantified and presented in a Table 2. While all the wild-type cells showed normal cell division, approximately 28% of *rapGAP9* null cells failed to undergo normal cell division. Further studies to test whether Rap1 activity and myosin assembly is altered in *rapGAP9* null cells during cytokinesis would be helpful for understanding RapGAP9-mediated cytokinesis.

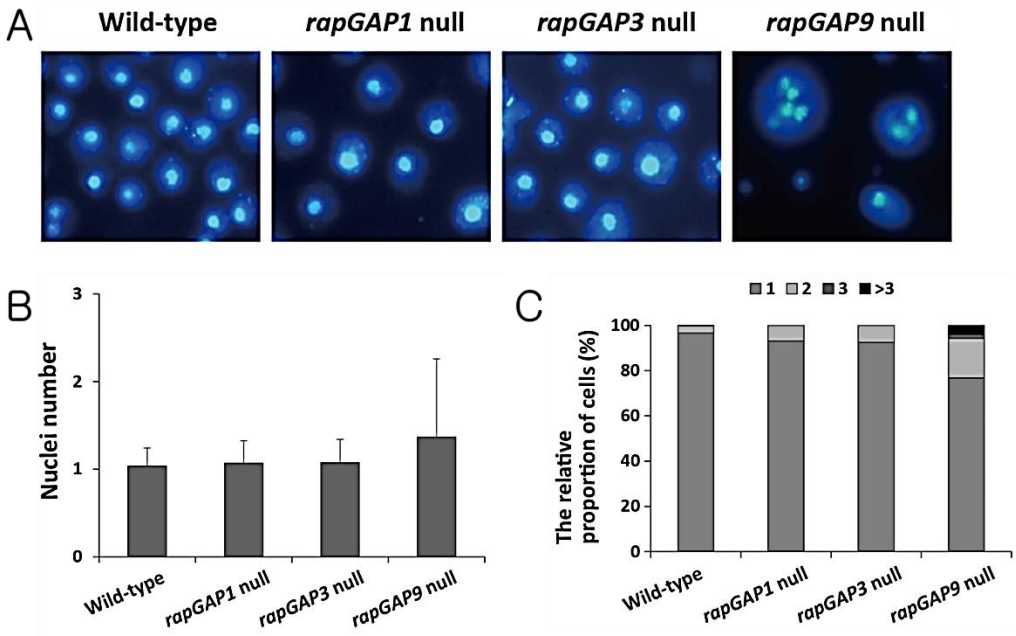



Fig.5. The cytokinesis of *rapGAPs* null cells.

(A) Nuclear staining. Cells were allowed to attach to plastic plate for 30 min. And then were stained with Hoechst dye. Representative DAPI images were shown. (B) Quantitative analysis of the number of nuclei in the cells. Error bars represent SD. (C) The relative proportion of the cell nuclei.

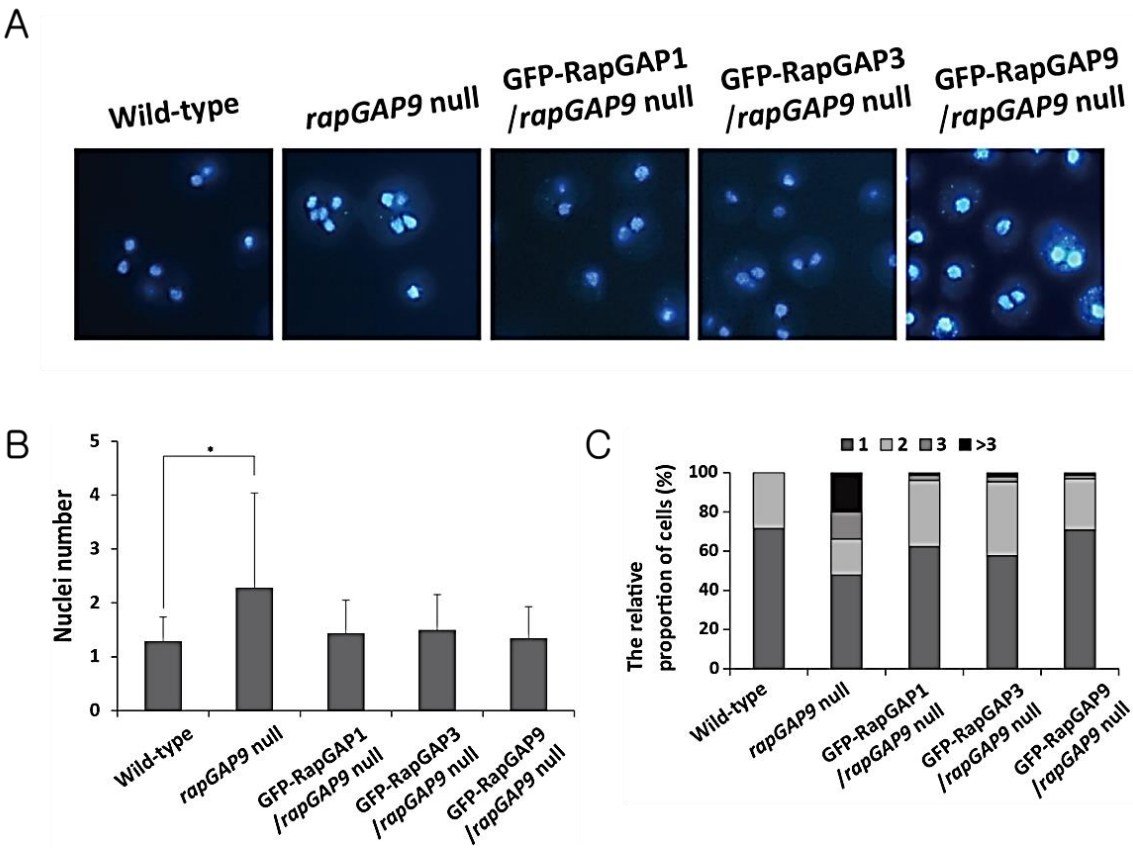



Fig.6. The cytokinesis of *rapGAP9* null cells expressing RapGAPs.

(A) Nuclear staining. Cells were grown in shaken suspension for 3 days and allowed to attach to plastic plates for 30 min. Representative DAPI images of the *rapGAP9* null cells expressing RapGAP proteins. (B) Quantitative analysis of the number of nucleus in the cells. Error bars represent SD. (C) The relative proportion of the cell nuclei. Statistically different from control at $^{*}p<0.05$ by the student's t-test.

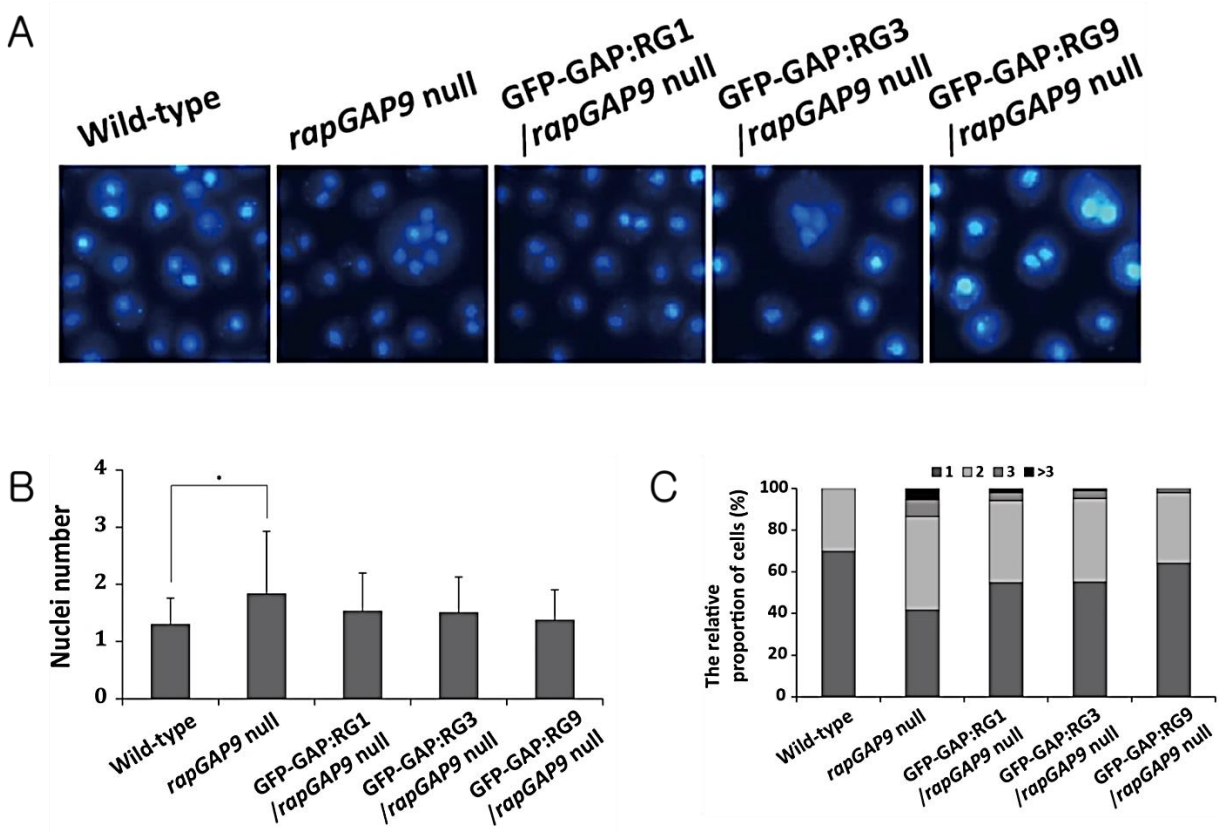
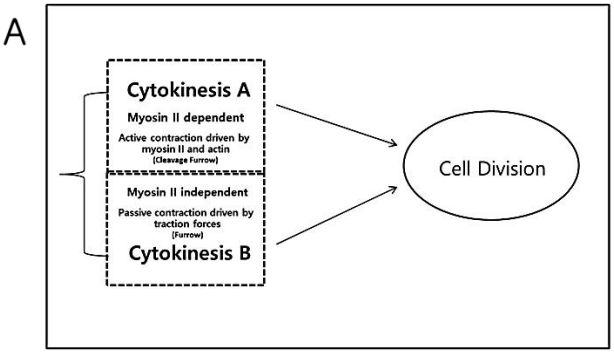
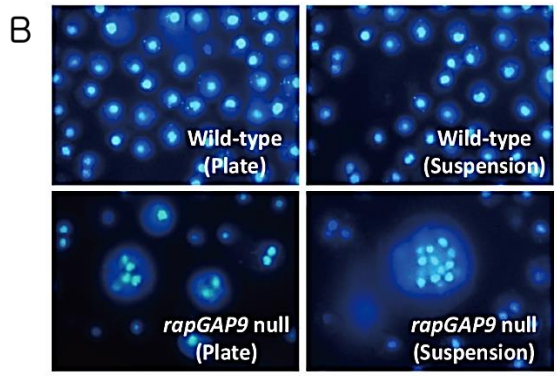
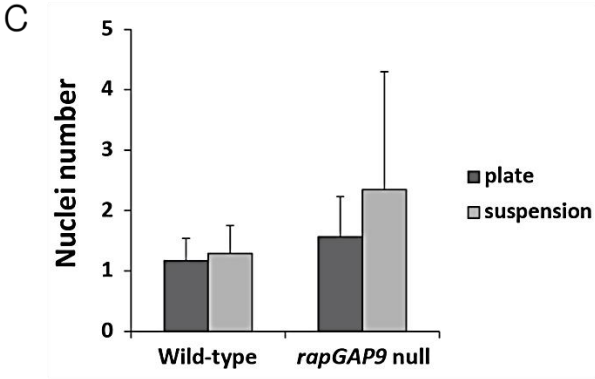


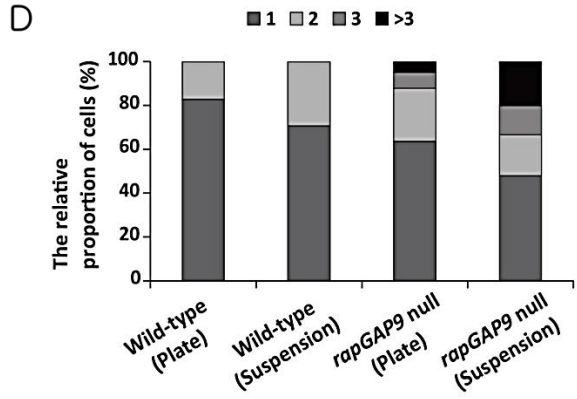

Fig.7. The cytokinesis of *rapGAP9* null cells expressing GAP domains of RapGAPs.

(A) Nuclear staining. Cells were allowed to attach to plastic plate for 30 min. Representative DAPI images of the *rapGAP9* null cells expressing GAPs. (B) Quantitative analysis of the number of nucleus in the cells. Error bars represent SD. (C) The relative proportion of the cell nuclei. Statistically different from control at $^{*}p<0.05$ by the student's t-test.



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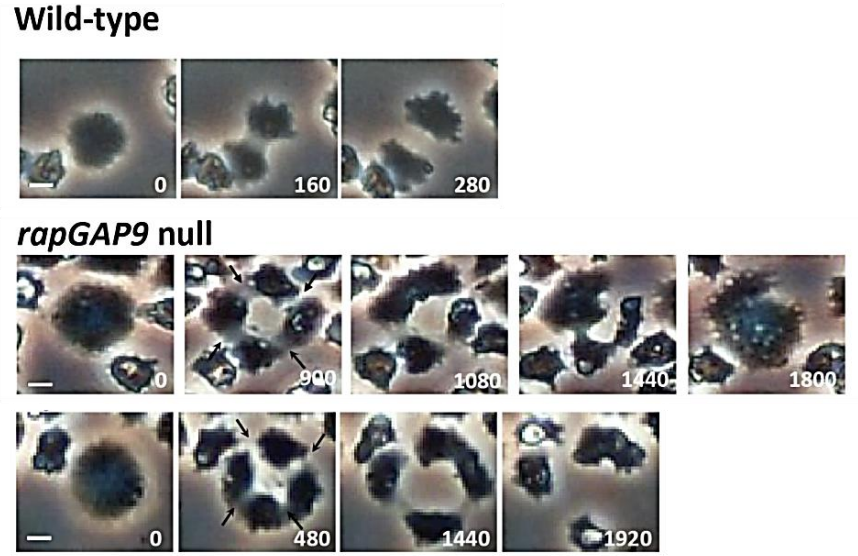



**Table 2. Cytokinesis phenotype in wild-type and *rapGAP9* null cells**

| Strain | Cytokinesis success (%) | Failure (%) | N |
|---|---|---|---|
| Wild-type | 100 | 0 | 40 |
| *rapGAP9* null | 72.09 | 27.90 | 43 |

Fig.8. Cytokinesis A and B defects of *rapGAP9* null cells.

(A) Nuclear staining. Cells were grown in shaken suspension for 3 days and allowed to attach to plastic plate for 20 min. Alternate panels showing cells stained with the Hochest dye, and phase contrast images. Schematic diagram of cell division in *Dictyostelium*. (B) Representative DAPI images. (C) Wild-type and *rapGAP9* null cells grown in cell culture dishes and in suspension, adhered to a 6 well plate for 30 min, and then stained. (D) The frequency of the nuclei on plate culture and in suspension culture. (E) Images of cell undergoing cytokinesis on substrates. Time in seconds is indicated. Arrows show the region where the furrow is being formed.

## III-4. Development

### 1) RapGAP3 is involved in the regulation of development

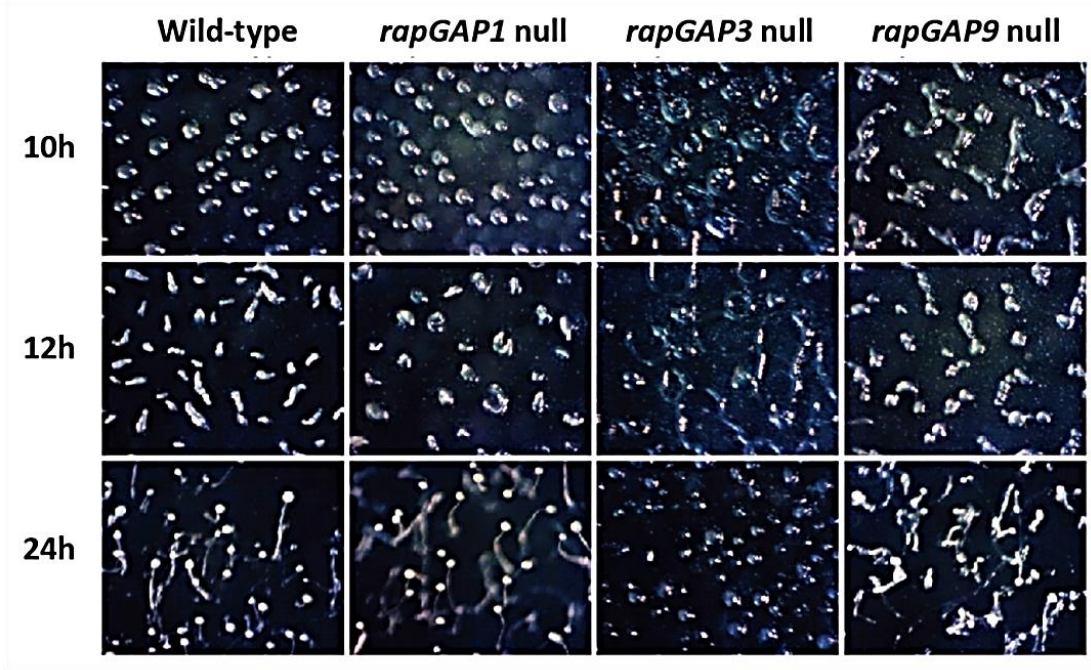
*Dictyostelium* cells release cAMP during development, causing surrounding cells to migrate and initiate the formation of a multicellular fruiting body (Jeon et al., 2009). To examine the possible roles of *rapGAP* proteins in development, I performed a development assay (Fig. 9A). Wild-type, *rapGAP1* null, and *rapGAP9* null cells showed normal development, compared to *rapGAP3* null cells which involved normal aggregation, formation of mound within 8 h, tip formation within 12 h, and finally, formation of the fruiting body. In contrary to other cells, *rapGAP3* null cells had loose aggregates, which were disaggregated in 10-12 h developmental precess, forming a ring-like structure, and also a delay in fruiting body formation. These results show that RapGAP3 is required for normal development.

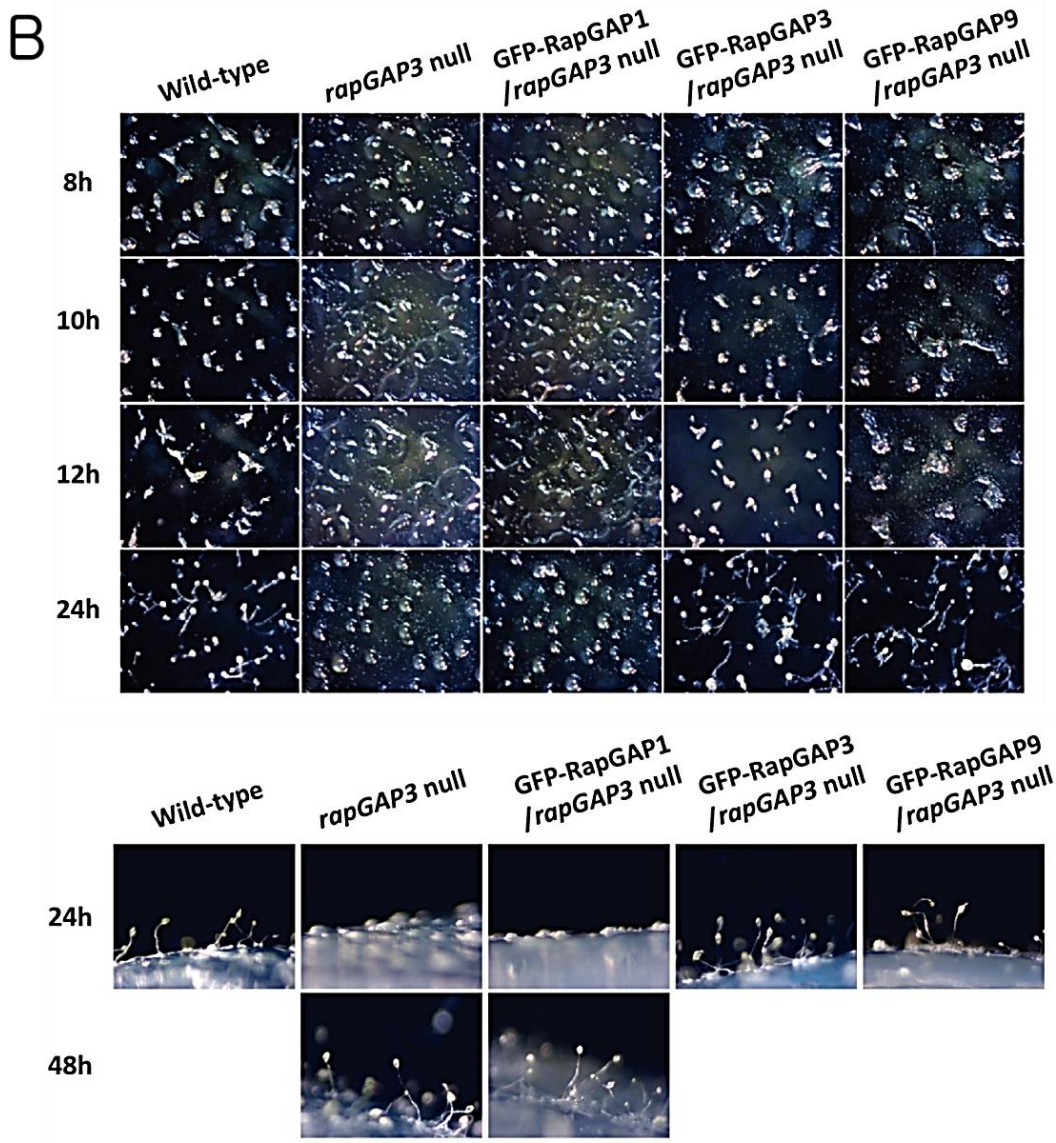
### 2) RapGAP3 and RapGAP9 have partially overlapped functions in development but not with RapGAP1

To determine if other RapGAP proteins could complement developmental defects of *rapGAP3* null cells, I examined and compared developmental phenotypes of wild-type, *rapGAP3* null cells, and *rapGAP3* null cells overexpressing RapGAP proteins or GAP-domain fragments. *rapGAP3* null cells expressing RapGAP1, as did *rapGAP3* null cells, formed a ring-structure at 10-12 h development and displayed highly delayed development finally

forming fruiting bodies. In contrast, *rapGAP3* null cells expressing RapGAP9 showed partially complemented phenotypes in development. These cells formed loose aggregates at 10–12 h development and showed no ring-structure, which were different from developmental phenotypes in *rapGAP3* null cell, and finally formed fruiting bodies at 24 h development, as shown in wild-type cells (Fig. 9B). *rapGAP3* null cells expressing GAP-domain fragments of RapGAP1 or RapGAP3 did not rescue the developmental phenotypes of *rapGAP3* null cells and showed ring-structures at 10–12 h development and delayed fruiting body formation. *rapGAP3* null cells expressing GAP-domain fragments of RapGAP9 formed loose aggregates at 10–12 h development and showed no ring-structure, suggesting that interestingly the GAP-domain of RapGAP9 has in part complementary functions in development (Fig. 9C). These results suggest that developmental defects of *rapGAP3* null cells were partially complemented by full-length RapGAP9 proteins and the GAP domain of RapGAP9, but not by full-length RapGAP1 or other GAP-domain fragments from other RapGAP proteins. Our results reveal that RapGAP3 and RapGAP9 have overlapped functions in development. It is noteworthy that the developmental phenotypes of *rapGAP3* null cells were rescued by the GAP-domain fragments of RapGAP9 but not by that of RapGAP3. These results raise a possibility that the activity of GAP domain in RapGAP3 might be modulated by interaction with other domains in RapGAP3 such as PH domains at the N-terminal.

A





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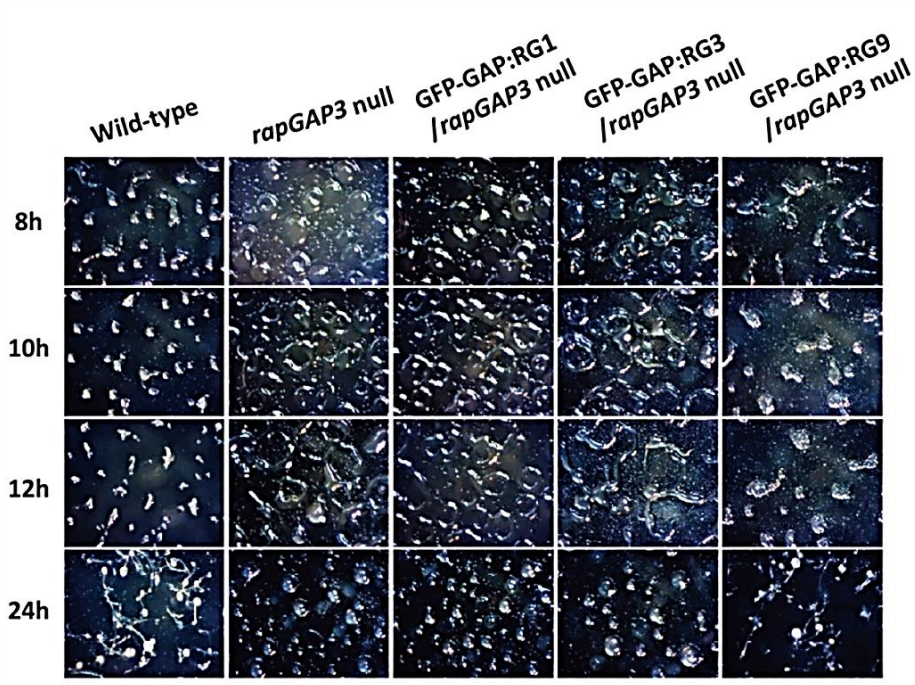


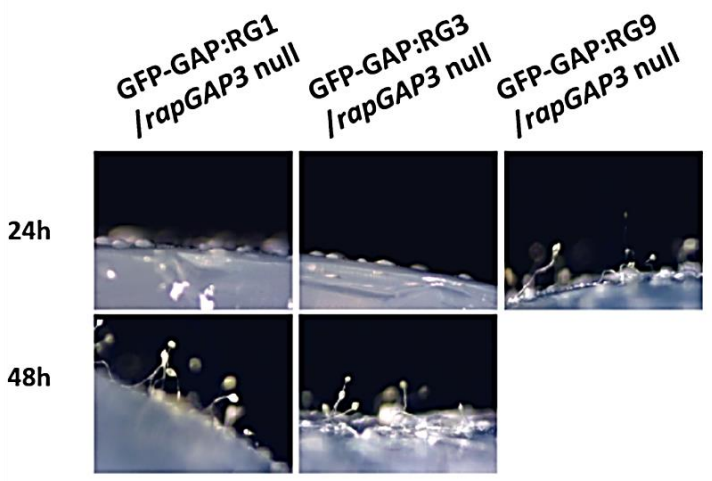

Fig.9. Development of *rapGAP3* null cells expressing RapGAPs or GAP domains of RapGAPs.

(A) Developmental morphology of *rapGAP1* null, *rapGAP3* null, and *rapGAP9* null cells. Vegetative cells were washed and plated on non nutrient agar plates. Photographs were taken at the times indicated after plating. Developmental images of the cells at 10 h (Wild-type early mound stage), 12 h (wild-type tip forming stage), 24 h (wild-type fruiting body stage) are shown. (B) Developmental morphology of *rapGAP3* null cell expressing RapGAPs. Development of *rapGAP3* null cells expressing RapGAPs and GAPs domains. Vegetative cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating. (C) Developmental morphology of *rapGAP3* null cells expressing GAPs-domain fragments. Side view of fruiting bodies. Development of *rapGAP3* null cells was delayed. The developmental morphologies of *rapGAP3* null cells at 24 h and 48 h development were compared with those of wild-type cells and the cells expressing RapGAPs and GAPs at 24 h and 48 h development.

## III-5. Adhesion

### 1) RapGAP1 and RapGAP9 involved in cell adhesion

To determine the possible roles of RapGAPs in cell adhesion, I investigated cell adhesion of *rapGAP* null cells by measuring the fraction of cells that attached from a plate during agitation. Compared to wild-type cells, *rapGAP1* null and *rapGAP9* null cells showed strong attachment (Fig. 10A). These results suggest that RapGAP1 and RapGAP9 play an important role in cell adhesion.

### 2) Adhesion defects of *rapGAP1* null cells were not complemented by overexpression of RapGAP3 and RapGAP9.

To determine if other RapGAPs could complement adhesion defects of *rapGAP1* null cells, I examined cell-substraum attachment of wild-type, *rapGAP1* null cells, and *rapGAP1* null cells overexpressing RapGAP proteins or GAP-domain fragments. *rapGAP1* null cells expressing RapGAP3 or RapGAP9, like *rapGAP1* null cells, showed strong adhesion. In addition, *rapGAP1* null cells expressing GAP-domain fragments of RapGAPs showed strong adhesion (Fig. 10B, Fig. 11A). These results suggest that adhesion defects of *rapGAP1* null cells were not complemented by full-length RapGAP3 or RapGAP9.

### 3) Adhesion defects of *rapGAP9* null cells were partially complemented by overexpression of RapGAP1 and RapGAP3.

To determine if RapGAP1 and RapGAP3 could complement adhesion defects of *rapGAP9* null cells, I measured and compared cell-substraum attachment of wild-type, *rapGAP9* null cells, and *rapGAP9* null cells overexpressing RapGAP proteins or GAP-domain fragments. Compared to *rapGAP9* null cells, *rapGAP9* null cells expressing full-length RapGAP proteins or GAP-domain fragments showed decreased cell-substratum adhesion, suggesting that the adhesion defects of *rapGAP9* null cells were partially complemented not only by full-length RapGAP1 and RapGAP3 but also by GAP-domain fragments of RapGAP1 and RapGAP3. These results are conflicted with those found in adhesion experiments using *rapGAP1* null cells. Examination of expression levels of the overexpressing cell lines and subcellular localization of the proteins would be required for further understanding the functions of RapGAP proteins in cell adhesion.

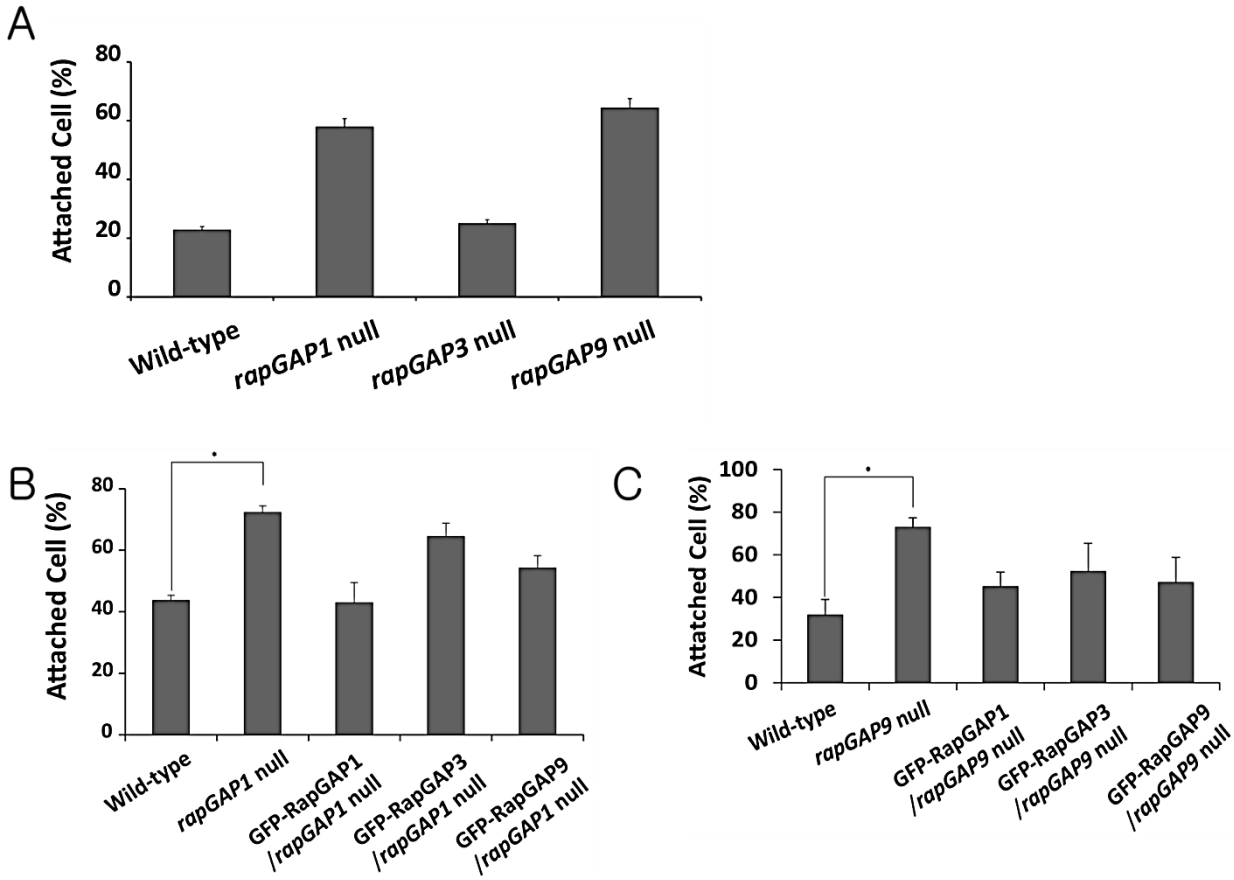


Fig.10. Cell adhesion of *rapGAP1* null and *rapGAP9* null cells expressing RapGAPs.

(A) Cell-substratum adhesion of Wild-type cells and *rapGAPs* null cells. (B) Cell-substratum adhesion of Wild-type, *rapGAP1* null cells, and *rapGAP1* null cells expressing RapGAPs. (C) Cell-substratum adhesion of Wild-type, *rapGAP9* null cells, *and rapGAP9* null cells expressing RapGAPs. Adhesion was measured by the ratio of attached cells to the total number of cells. Experiments were performed at least three times. Error bars represent SE. Statistically different from control at $^{*}p<0.05$ by the student's t-test.

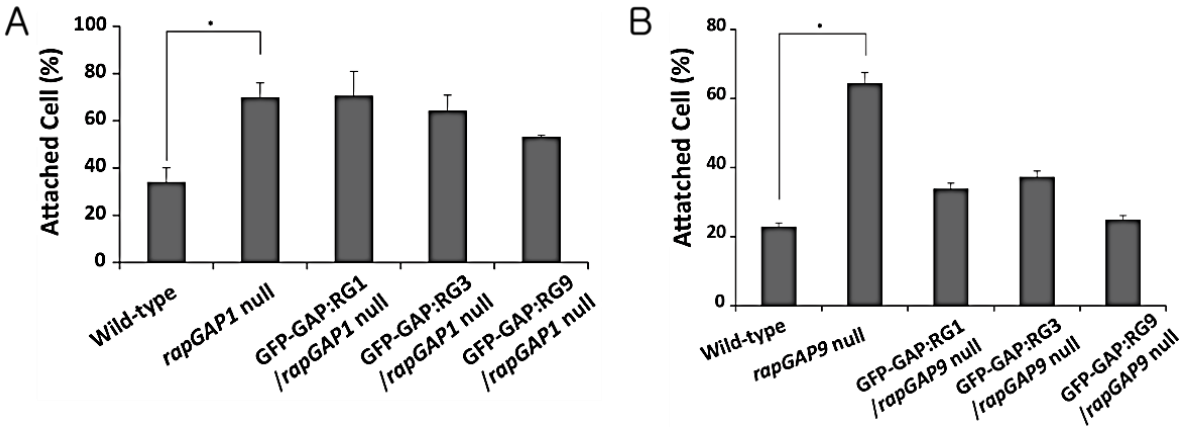


Fig.11. Cell adhesion of *rapGAP1* null and *rapGAP9* null cells expressing GAP domains of RapGAPs.

(A) Cell-substratum adhesion of Wild-type, *rapGAP1* null cells, and *rapGAP1* null cells expressing GAPs. (B) Cell-substratum adhesion of wild-type, *rapGAP9* null cells, and *rapGAP9* null cells expressing GAPs. Adhesion was measured by the ratio of attached cells to the total number of cells. Experiments were performed three times. Error bars represent SE. Statistically different from control at $^{*}p<0.05$ by the student's t-test.

# IV. Discussion

## Morphology & Cell adhesion

Functions of RapGAPs regulating Rap1 activity were investigated in the present study. It has been reported that RapGAP1 and RapGAP3 are involved in various cellular functions by controlling Rap1 activity. Specific or common functions of RapGAP proteins are presented in Table 3. In this study, first I examined the phenotypes of *rapGAP1*, *rapGAP3*, and *rapGAP9* null cells in various biological processes including morphogenesis, cell adhesion, development, cytokinesis, and then investigated whether these phenotypes of *rapGAP* null cells were rescued by expressing each RapGAP protein or GAP-domain fragments from RapGAP proteins. *rapGAP9* null cells were flat and abnormally large in size. In addition, they had a strong cell substratum adhesion, which was similar to the cells expressing constitutively active Rap1 (Rap1G12V) or overexpressing Rap1-specific GEF protein (Kortholt and van Haastert, 2008). These observations suggest that *rapGAP9* null cells might have a higher Rap1 activity in vegetative cell state, and I confirmed that *rapGAP9* null cells have high level of Rap1 activity compared to wild-type cells (data not shown). The morphological defects of *rapGAP9* null cells was rescued by expression of RapGAP1 or RapGAP3. It seems that high levels of Rap1 activity in *rapGAP9* null cells decreased to the normal levels by RapGAP1 or RapGAP3 in *rapGAP9* null cells expressing RapGAP1, RapGAP3, or GAP-domain fragments.

I investigated the overlapping/specific functions of RapGAP1, RapGAP3, and RapGAP9 in cell adhesion. Rap1 is involved in integrin-mediated cell adhesion

and modulates integrin affinity (Webb et al., 2002) . In *Dictyostelium*, Rap1 is a key molecule controlling cell adhesion (Jeon et al., 2007b). *rapGAP1* null cells and *rapGAP9* null cells exhibit strong adhesion to the substratum as observed in cells expressing constitutively active Rap1G12V (Jeon et al., 2007b). These phenotypes in *rapGAP9* null cells were partially complemented by expressing RapGAP1 or RapGAP3, whereas abnormally strong cell adhesion of *rapGAP1* null cells was not complemented by overexpression of RapGAP3 or RapGAP9. Further confirmation experiments are required.

Table 3. Overview of complementary functions of RapGAP proteins in *Dictyostelium*

| Cell | Insert protein | Morphology | Adhesion | Cytokinesis | Development |
|---|---|---|---|---|---|
| ***rapGAP1* null** | - | - | **Strong attachment** | - | - |
| | RapGAP1 | - | Complement | - | - |
| | GAP: RapGAP1 | - | Not complement | - | - |
| | RapGAP3 | - | Not complement | - | - |
| | GAP: RapGAP3 | - | Not complement | - | - |
| | RapGAP9 | - | Not complement | - | - |
| | GAP: RapGAP9 | - | Not complement | - | - |
| ***rapGAP3* null** | - | - | - | - | **Ring structure, Loose aggregates, Delay fruiting body formation** |
| | RapGAP1 | - | - | - | Not complement |
| | GAP: RapGAP1 | - | - | - | Not complement |
| | RapGAP3 | - | - | - | Complement |
| | GAP: RapGAP3 | - | - | - | Not complement |
| | RapGAP9 | - | - | - | Partially Complement |
| | GAP: RapGAP9 | - | - | - | Partially Complement |
| ***rapGAP9* null** | - | **Increased cell size** | **Strong Attachment** | **Multinucleated** | **Delay fruiting body formation** |
| | RapGAP1 | Partially complement | Complement | Multinucleated /partially complement | - |
| | GAP: RapGAP1 | Partially complement | Partially complement | Multinucleated /partially complement | - |
| | RapGAP3 | Partially complement | Partially complement | Multinucleated/ partially complement | - |
| | GAP: RapGAP3 | Partially complement | Partially complement | Multinucleated/ partially complement | - |
| | RapGAP9 | Complement | Complement | Complement | Complement |
| | GAP: RapGAP9 | Complement | Complement | Complement | Complement |

## Cytokinesis & Development

It is known that cell division in *Dictyostelium* is mediated by two different types of cytokinesis pathways (Nagasaki et al., 2009). The cytokinesis type B is independent of Myosin II, and the mutant cells in the cytokinesis type B pathway produce giant multinuclei on plate culture. In the myosin II dependent cytokinesis type A, the mutant cells divide normally on plate culture, but are unable to divide at all and become highly multinucleated in suspension culture (Robinson et al., 2002). Any mutant cells with a defect in cytokinesis type B would show giant and multinucleated on plate culture, while mutant cells with a defect in cytokinesis type A would display abnormal cell division in suspension culture (De Lozanne and Spudich, 1987). *rapGAP9* null cells were multinucleate on plate culture. In suspension culture, *rapGAP9* null cells showed slightly increased cell sizes and the number of nuclei compared to those on plate culture. These results suggest that RapGAP9 is involved in the precess of both cytokinesis type A and B. Multinucleate phenotype of *rapGAP9* null cells indicates that RapGAP9 should be involved in cytokinesis. In this study, I proved that cytokinesis defects of *rapGAP9* null cells were partially complemented by RapGAP1 and RapGAP3. A recent study demonstrated that constitutively active Rap1 caused cytokinesis defect. In *Dictyostelium*, Rap1 controls cytokinesis process through regulation of cytoskeletal components. Constitutively active Rap1 regulates cytoskeletal components, leading to furrow regression and asymmetric cell division (Plak et al., 2014). Therefore, it seems that high Rap1 activity of *rapGAP9* null cells was influenced by overexpression of RapGAP1 and RapGAP3, which partially affected regulation of cytoskeletal components, resulting in reduction of the proportion of

multinucleate cells.

Multicellular development of *Dictyostelium* is initiated by starvation, during which cAMP pulse is generated in cells and is transduced towards the periphery (Loomis, 2014). cAMP acts as a chemoattractant and induces chemotaxis, resulting in aggregation and mound formation (Chisholm and Firtel, 2004). This eventually culminates in multicellular fruiting body formation through regulation by extracellular cAMP phosphodiesterases (Parkinson et al., 2009). The Rap1 signaling pathway is important for this process. It has been revealed that RapGAP3 and RapGAPB regulate cell morphology during multicellular development. Recently, it was also reported that RapGAP9 was essential for normal fruiting body formation in development and was involved in chemotaxis (Mun et al., 2014). In this study, I proved that developmental defects of *rapGAP3* null cells were partially complemented by RapGAP9 but not by RapGAP1. Presumably, this result seems to be related to the activity levels of Rap1 in development steps. RapGAP3 is involved in late aggregation stage of development, and RapGAP9 is likely to be involved in late stage of development (fruiting body stage).

## Conclusion

The small GTPase Rap1 is involved in the dynamic control of cellular processes, integrin-mediated cell adhesion, cell migration in *Dictyostelium*. RapGAPs are specific GAP proteins for Rap1 and regulate Rap1 activity in diverse cellular phenomena. In morphogenesis and cytokinesis, RapGAP proteins have partially overlapped functions. RapGAP3 and RapGAP9 appear

to share their functions in developmental process. Identification of overlapping/specific functions of RapGAP1, RapGAP3, and RapGAP9 in this study will provide further insights into the molecular mechanism through which Rap1 controls cell adhesion, cytokinesis, and development.

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