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Opposite Functions of RapA and RapC and Roles of RasZ in *Dictyostelium*

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딕티오스텔리움에서 RapA와 RapC의 상반된 기능과 RasZ의 기능

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이 논문을 이학석사학위 신청 논문으로 제출함

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I. Opposite Functions of RapA and RapC in Cell Adhesion and Migration in *Dictyostlium*.

PART II. Roles of RasZ Protein in *Dictyostelium* Cell Migration and Development

ABBREVIATIONS

CA	Constitutively active
cAMP	Cyclic adenosine monophosphate
cAR	Cyclic AMP reporters
DAPI	4', 6'-diamidine-2'-phenylidole dihydrochloride
DN	Dominantly negative
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
OE	Over-expressing
PI3K	Phosphatidylinositol 3-kinase
SD	Standard deviation
SEM	Standard error of measurement
TRITC	Tetramethylrhodamine

ABSTRACT

Opposite Functions of RapA and RapC and Roles of RasZ in *Dictyostelium*

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Ras proteins, small GTPases, are major regulators of diverse signaling pathways including cell migration, adhesion, and development. 15 Ras proteins are found in *Dictyostelium* genome. Rap proteins among them play an important role in cell adhesion and cell migration. Recent studies suggested that RapA and RapC had an antagonistic effect on cell spreading, cell adhesion, and cell migration. Here, I investigated the opposite functions of RapA and RapC in cellular processes. First, I compared the phenotypes of RapA or RapC overexpressing cells in cell morphology, cell adhesion, cell migration, and development. RapA overexpressing cells exhibited highly spread morphology, strong adhesion and slow migration speed. In contrast, RapC overexpressing cells showed shrank and rounded morphology, weak adhesion and accelerated migration speed compared to wild-type cells, which are opposite phenotypes to RapA. *rapC* null cells showed similar phenotypes to RapA overexpressing cells, such as spread morphology, strong adhesion and slow migration speed. Reintroduction of RapC into *rapC* null cells rescued the phenotypes in cell morphology and cell adhesion. RapC appears to play a unique role in multicelluar developmental process. *rapC* null cells had a defect in development by forming the abnormal multiple tips from one mound. In this

study, I confirmed that RapA and RapC have opposite functions in the regulation of the cell size, cell attachment to substrate, and cell migration during chemotaxis. In the domain structure, RapC has additional amino acids at the C-terminus, which are not found in RapA. The mechanisms for opposite functions of RapA and RapC in various cellular processes is required further to understand the Rap signaling pathways.

Cell migration is essential for immune response, wound healing, and metastasis of cancer. The defects in cell migration are not only associated with cancer, but also with many other diseases such as rheumatism and osteoporosis. Ras proteins have been studied over 4 decades because of their crucial roles in human oncogenesis. It also has been known that Ras proteins play an important role in the regulation of cytoskeleton during cell migration. The Ras GTPase subfamily in *Dictyostelium* is consisted of 15 proteins; 11 Ras, 3 Rap, and one Rheb related protein. Rap1 has been the most studied of the Ras family because it is known as the antagonist of Ras protein. Rap1 plays important roles in integrin-mediated cell adhesion, cadherin-based cell-cell adhesion, and cell polarity in mammalian cells. In *Dictyostelium*, Rap1 is involved in controlling call adhesion, phagocytosis, and cell migration. Despite of the importance of Ras family in cellular processes, the functions of many Ras proteins remain unknown. Here I examined the roles of RasZ in morphogenesis, cell adhesion, cell migration, and multicellular development. Phylogenetic analysis showed that RasZ belongs to a group with RasX and RasW, showing 90% and 82% identities respectively. To investigate the roles of RasZ, I compared the phenotypes of wild-type cells, *rasZ* null cells, and *rasZ* null cells expressing RasZ. *rasZ* null cells showed small cell size and smooth cell surface, and having weak cell adhesion to the substratum, comparing with wild-type cells. Cells lacking RasZ also showed increased migration during chemotaxis and small size of spores and slightly elongated stalks during multicellular development. These phenotypes were rescued by expressing of RasZ. This study shows that RasZ plays an important role in diverse cellular

processes including morphogenesis, cell adhesion, migration, and development in *Dictyostelium*.

국문초록

딕티오스텔리움에서 RapA와 RapC의 상반된 기능과 RasZ의 기능

전 지 현

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작은 GTPase인 Ras 단백질은 세포 이동 및 부착 그리고 발생과정을 포함한 다양한 신호 전달 경로의 주요 조절자이다. 15개의 Ras 단백질이 딕티오스텔리움 게놈에서 밝혀졌다. Ras 단백질 중 Rap 단백질은 세포의 부착과 이동에 중요한 역할을 한다. 최근 연구결과에 따르면 RapA와 RapC가 세포 형태, 세포 부착력 그리고 세포 이동에서 상반된 기능을 보였다. 먼저, 과발현된 RapA와 RapC의 세포 형태, 세포 부착, 세포 이동 그리고 발생과정에서의 표현형을 비교하였다. 야생형 세포와 비교하였을 때, 과발현된 RapA 세포는 증가된 세포크기와 기질과의 강한 부착력을 보였으며 세포이동속도가 감소되었다. 반면, 과발현된 RapC 세포는 세포의 표면이 둥글며 줄어든 세포크기와 약한 부착력을 보였고 세포이동 속도가 증가되었다. *rapC* null 세포는 과발현된 RapA와 같이 증가된 세포형태, 강한 세포 부착력 그리고 느린 세포이동 속도를 보였다. RapC를 삽입한 *rapC* null세포는 *rapC* null세포가

가진 세포 크기와 부착력에 대한 결함을 보완하였다. 놀랍게도 다세포 발생 과정에서 RapC는 독특한 역할을 보였다. *rapC* null 세포는 한 개의 마운드에서 비정상적으로 여러 개의 자실체 줄기를 형성하는 결함이 있었다. 본 연구를 통해 딕티오스텔리움 내 RapA와 RapC가 세포 크기, 부착력 및 세포이동 속도를 반대로 조절한다는 것을 확인했다. 도메인 구조에서 RapC는 RapA에서 찾을 수 없던 추가 아미노산 서열을 C말단에 보유했다. RapA와 RapC의 다양한 세포 과정에서 상반된 기능에 대한 메커니즘은 추후 Rap 신호전달을 이해하기위해 요구된다.

세포이동은 면역반응, 상처 치유, 그리고 암의 전이에 중요하다. 세포이동 조절의 실패는 암뿐만 아니라, 골다공증, 생명현상 유지의 필수적인 현상이다. Ras 단백질은 사람의 종양 형성에 중요한 역할을 하기 때문에 지난 40년간 연구되어 왔다. 또한 Ras 단백질은 세포이동 동안 세포골격을 조절하는데 중요한 역할을 한다. 딕티오스텔리움에서 Ras GTPase 아과는 11개의 Ras, 3개의 Rap, 그리고 1개의 Rheb 연관 단백질로 총 15개의 Ras 단백질로 구성되어 있다. Rap1은 Ras 단백질의 길항자로 잘 알려져 있기 때문에 Ras 과 중 가장 많이 연구되어 왔다. Rap1은 척추동물 세포에서 인테그린 연관 세포의 부착, 카드헤린 기반 세포들 간 부착, 그리고 세포의 분극화에 중요한 역할을 한다. 딕티오스텔리움에서 Rap1은 세포부착, 식세포작용, 그리고 세포이동의 조절을 담당한다. Ras 과 단백질의 중요성에도 불구하고 많은 Ras 단백질은 아직까지 잘 알려져 있지 않다. 그러므로 딕티오스텔리움을 이용해 Ras 단백질인 RasZ의 세포형태, 세포부착, 세포 이동, 그리고 다세포 발생과정에 어떤 역할을 하는지 본 연구를 통하여 알아보고자 한다. 계통수 분석에서 RasZ는 RasX와 90%, RasW와 82%의 높은 상동성을 보였다.

RasZ의 기능에 대해 알아보기 위해 야생형 세포, *rasZ* null 세포, 그리고 RasZ를 삽입한 *rasZ* null 세포의 표현형을 비교했다. *rasZ* null 세포는 야생형과 비교했을 때, 세포의 크기가 작았고 기질과의 약한 부착력을 보였으며, 주화성이동동안 증가된 세포이동속도를 보였다. 다세포 발생과정 동안, *rasZ* null 세포는 줄기의 길이가 길어졌으며 포자체의 크기가 감소했다. 이러한 표현형은 RasZ에 의해서 보완되었다. 연구를 통하여 RasZ가 딕티오스텔리움 내에서 세포의 형태와 기질과의 부착력 및 세포이동속도를 조절함을 확인하였다. 본 연구를 통해 딕티오스텔리움에서 RasZ가 세포형태, 기질과의 부착력 및 세포이동, 그리고 발생과정을 포함한 여러 세포 과정을 조절함을 확인했다.

❖ This part is submitted for publication as Jihyeon Jeon and Taeck Joong Jeon, 2020.

Part I. Opposite functions of RapA and RapC in cell adhesion and migration in *Dictyostelium*

I. INTRODUCTION

Ras proteins which have been studied for 40 years regulate cellular signaling pathways, such as cell morphology, migration, cell survival, cell cycle progression, and endocytosis. The Ras superfamily have been identified more than 150 members in mammalian genomes and is divided into 5 subfamilies: RAS, RHO, RAB, ARF, and G α subunits of heterotrimeric G-proteins (Guo et al., 2016; Wennerberg et al., 2005). Ras family is composed of 3 Ras (H-Ras, K-Ras, and N-Ras), 5 Rap (Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C), R-Ras, Ral, and Rheb proteins, and are small monomeric GTPases that cycle between GTP-bound activated and GDP-bound inactivated forms by GEFs (Guanine nucleotide effector factors) and GAPs (GTPases activating proteins), respectively (Weeks and Spiegelman, 2003) . Those forms of RAS are affected by GEFs and GAPs when they are assembled at the plasma membrane and located in the vicinity of RAS (Simanshu et al., 2017).

Dictyostelium discoideum, free-living social amoeba, has been studied for understanding directional cell movement towards chemoattractants, cellular signaling and interaction including Ras signaling processes (Eichinger et al., 2005; Lee and Jeon, 2012). To perform the normal chemotaxis, chemoattractants should bind to G-protein coupled receptors which locates at cell surface. Identified cAMP receptors (cAR) are cAR 1, 2, 3,

and 4 in *Dictyosteilum*. 4 cAMP receptors (cAR) have been identified in *Dictyostelium*. Among the identified receptors, cAR1 has a high affinity with cAMP. It is critical for the early multicellular development and cell motility towards chemoattractant. Other cAR receptors (cAR 2-4) have low affinity with cAMP, but also essential for the multicellular development.

It is also great model organism for studying Ras signaling pathways, which contains conserved orthologs of the Ras superfamily, along with *Drosophila, C. Elegans, S. cerevisiae*, and *S. pombe* (Wennerberg et al., 2005). Ras family is consisted of 15 proteins in *Dictyostelium*: 11 Ras (RasB, RasC, RasD, RasG, RasS, RasU, RasV, RasW, RasX, RasY, and RasZ), 3 Rap (RapA, RapB, and RapC), and 1 Rheb-related protein (Park et al., 2018). Ras proteins in *Dictyostelium* are associated with various cellular processes, such as cell motility, cell polarity, cytokinesis, chemotaxis, micropinocytosis, and multicellular development (Kortholt et al., 2006).

Rap proteins are known as Ras antagonist and showed 50~60% sequence similarities with the product of Ras proto-oncogene (Park et al., 2018; Qu et al., 2016). They are consisted of several isoforms: 2 Rap1 (Rap1A and Rap1B) and 3 Rap2 (Rap2A, Rap2B, and Rap2C). Rap proteins function in multiple key signaling pathways, such as the regulation of integrin-mediated cell adhesion, cell-cell junction formation, establishment of cell polarity, exocytosis, cell apoptosis, carcinogenesis, and cell proliferation in mammalian cells (Guo et al., 2016).

RapA plays a major role in cell growth, proliferation, and cell survival in mammalian cells (Hilbi and Kortholt, 2019; Jeon et al., 2007). In addition, RapA is related to cytoskeletal regulation, phagocytosis, and the response to osmotic stress in *Dictyostelium* (Jeon et al., 2007). Cells overexpressing RapA showed spread morphology, increased cell adhesion with matrix, decrease of cell movement to extracellular chemoattractant, and delayed formation of fruiting bodies during multiple cellular development (Lee and Jeon,

2012). Loss of RapC (*rapC* null cells) exhibited enlarged shape of cells, formation of multinucleated cells resulting the defects in cytokinesis, increase of cell attachment, decreased chemotaxing migration speed, and formation of multiple tips from 1 mound during development (Park et al., 2018).

II. MATERIALS AND METHODS

II-1. Strains and Cell culture

Dictyostelium wild-type KAx-3 cells were grown in germless HL5 medium or in conjunction with *Klebsiella aerogenes* at 22°C. Knock-out strains and transformants were preserved in 10 μg/mL blasticidin and 10 μg/mL G418. The coding sequence of *rapA* cDNA was obtained by RT-PCR and was cloned into the *Eco*RI – *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. Transformant strains, constitutively active form of *rapA* (GFP-RapA^{G14V}) and dominantly negative form of *rapA* (GFP-RapA^{S19N}), *rapA* sequences were amplified by PCR using site-directed mutagenesis and was cloned into the *Eco*RI – *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment (Jeon et al., 2007). The coding sequence of *rapC* cDNA was obtained by RT-PCR and was cloned into the *Eco*RI – *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. Transformant strains, constitutively active form of *rapC* (GFP-RapC^{G13V}) and dominantly negative form of *rapC* (GFP-RapC^{S18N}), *rapC* sequences were amplified by PCR using site-directed mutagenesis and was cloned into the *Eco*RI – *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment. *rapC* knockout construct was generated by inserting the blasticidin resistance cassette (*bsr* cassette) into *BamHI* site which was created at the nucleotide 535 of *rapC* gDNA and used for the replacement of gene in KAx-3. (Park et al., 2018)

II-2. Cell adhesion assay

Cell adhesion assay was performed as described previously (Kim et al., 2017). Exponentially growing cells on the plates were washed with 12 mM Na/K phosphate buffer (pH 6.1) and the amounts of 2×10^6 cells in 200 μl were placed on the 6 well culture dishes for 20 min. Before shaking the plates, the attached cells were photographed and counted for calculating the total cell number. To quantify the adhered cell number, plates were then shaken at 150 rpm for 1h followed by washing off the detached cells. The number of attached cells in 6 well plate was counted. Cell attachment was presented as a percentage of attached cells compared with total cells.

II-3. DAPI staining

Log-phase growing cells were prepared on the coverslip and fixed with 3.7 % formaldehyde for 20 min. The fixed cells were washed by using PBS buffer (pH 7.0), were permeabilized with 0.1 % Triton X-100, drying, and staining with 3μg/mL Hoechst dye in 1 mL of mounting solution Fluoromount-G (SouthernBiotech). The epifluorescence images were captured by using NIS-elements software.

II-4. Chemotaxis assay

Chemotaxis toward cAMP was performed as described previously (Park et al., 2018). Aggregation-competent cells were prepared by incubating the cells at a density of 5×10^6 cells/mL in 1× Na/K phosphate buffer for 10h. Cell migration towards the chemoattractant, cAMP, was analyzed by using a Dunn chemotaxis chamber (Hawksley). Images of chemotaxing cells towards cAMP were obtained at time-lapse intervals of 1 min for 1h using an inverted microscope with a camera (Nikon). The data of chemotaxis were analyzed using NIS-Elements software (Nikon) and ImageJ software.

II-5. Development assay

Development was performed as described previously (Park et al., 2018). Log-phase growing cells were harvested and washed twice with 1× Na/K phosphate buffer, and then plated on Na/K phosphate agar plates at a density of 3.5×10^7 cells/mL. The multicellular development stages of the cells were examined by photographing the developing cells with a phase-contrast microscope at the times indicated in the figures.

Table 1. Primers used in the RapC point-mutation experiments.

primer	Sequence (5′ → 3′)
RapC CA - F	GTTTTGGGCGCAAGTGTTACTGG
RapC CA - R	CCAGTAACACTTGCGCCCAAAAC
RapC DN - F	GGTAAGACTAATTTAACTGTTAGATTTG
RapC DN - R	CAAATCTAACAGTTAAATTAGTCTTACC

F: Forward, R: Reverse

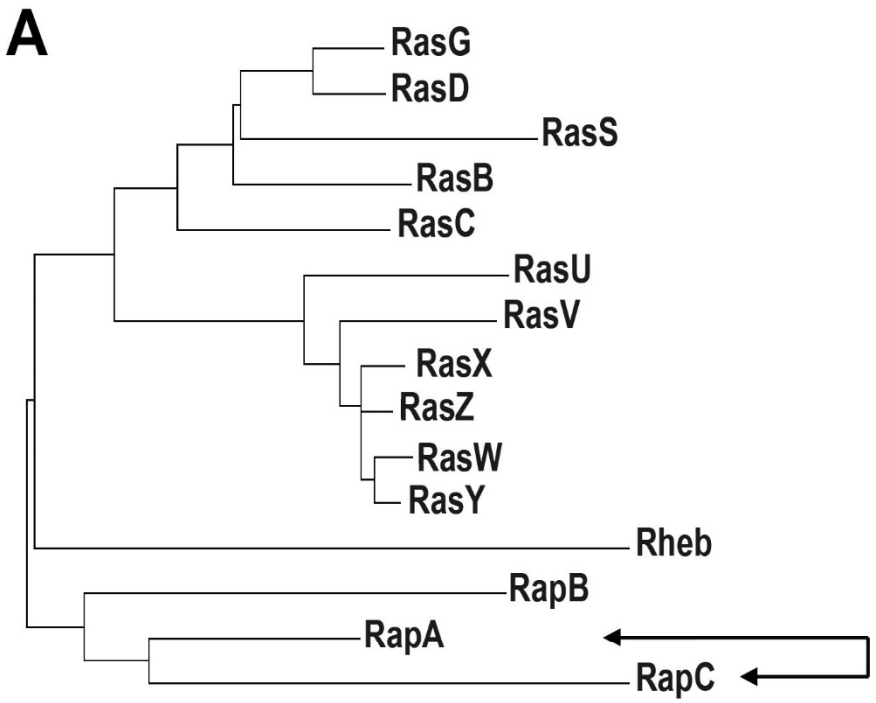
CA: Constitutively active, DN: Dominantly negative

III. RESULTS

III-1. Phylogenetic analysis of RapA and RapC.

There are three Rap (RAs-Proximate) proteins within *Dictyostleium*, and are composed of RapA, RapB, and RapC. Cells overexpressing RapA and RapC have been studied in diverse cellular processes.

To identify how RapA and RapC could function adversely in *Dictyostelium*, I investigated a computer analysis. The phylogenetic tree was drawn by comparing the amino acids for Ras domains of Ras proteins (Fig. 1A). RapA and RapC showed high similarity in the phylogenetic tree and showed 51% identities. RapA and RapC are composed of 186 amino acids and 278 amino acids respectively and both proteins have RAS domain at the N-terminus. RapC has additional amino acids residue at the C-terminus (Fig. 1B). To verify the functions of RapC, cells overexpressing GFP-RapC, constitutively active form of RapC, and dominantly negative form of RapC (expected molecular mass of 57 kDa) were prepared, and the expression of the protein was examined by western blotting using anti-GFP antibodies (Fig. 2B).



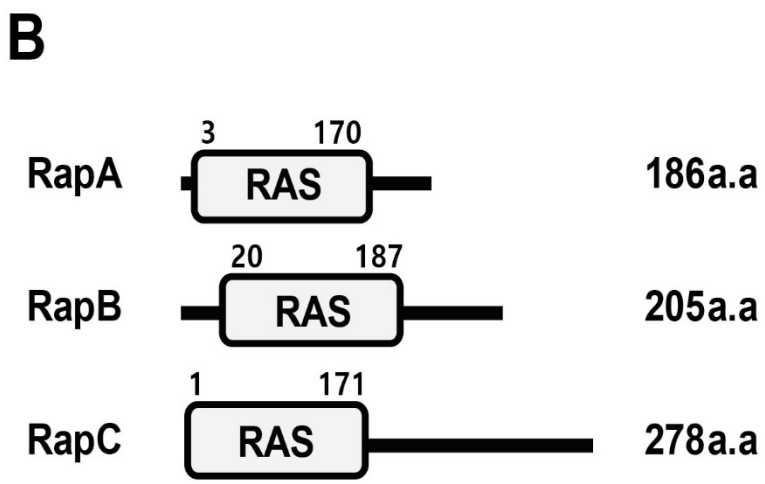


Fig 1. Phylogenetic tree of Ras proteins and domain structures of Rap proteins in *Dictyostelium.*

(A) Phylogenetic tree analysis of RAS proteins in *Dictyostelium*: RapA(dictyBase ID: DDB_G0216229), RapB(dictyBase ID: DDB_G0272857), RapC(dictyBase ID: DDB_G0272857DDB_G0270340), RasB(dictyBase ID: DDB_G0292998), RasC(dictyBase ID: DDB_G0281385), RasD(dictyBase ID: DDB_G0292996), RasG(dictyBase ID: DDB_G0293434), RasS(dictyBase ID: DDB_G0283537), RasU(dictyBase ID: DDB_G0270138), RasV(dictyBase ID: DDB_G0270736), RasW(dictyBase ID: DDB_G0270122), RasX(dictyBase ID: DDB_G0270124), RasY(dictyBase ID: DDB_G0270126), RasZ(dictyBase ID: DDB_G0270140), cpras1(dictyBase ID: DDB_G0277381), Rheb(dictyBase ID: DDB_G0277041), rsmA(dictyBase ID: DDB_G0283547), rsmB(dictyBase ID: DDB_G0281253), and DDB G0280437. The phylogenetic tree was drawn by MEGA7 to see the homolog among RAS domains. (B) Domain structures of RapA and RapC in *Dictyosteilum* indicating RAS domain at the N-terminus (RapA: 3-170 a.a and RapC: 1-171 a.a). RapC has an additional stretch of amino acids at the C-terminus.

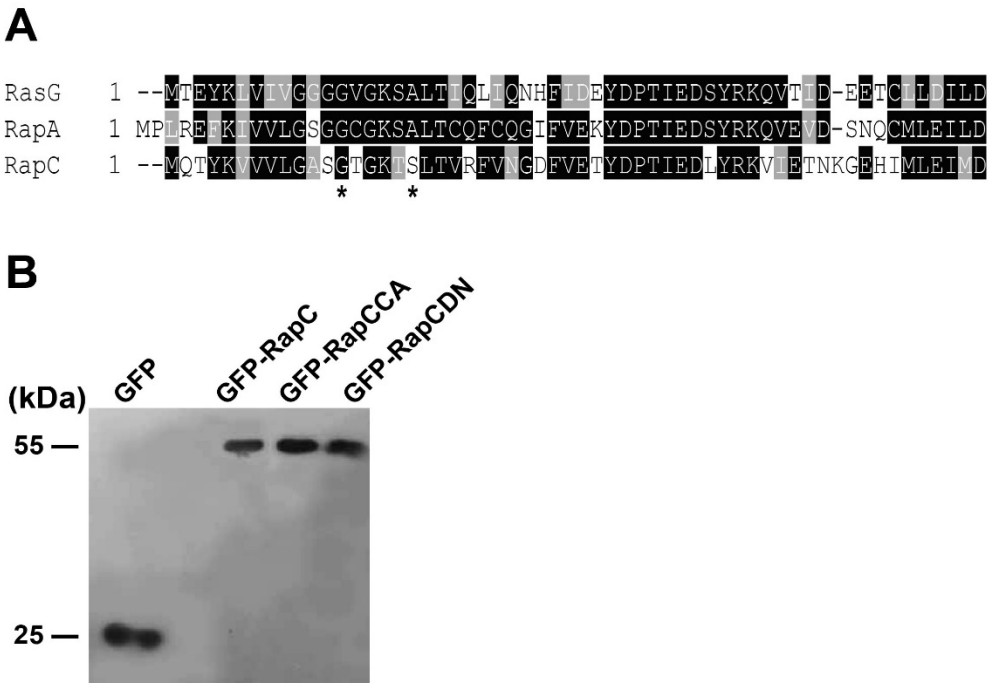


Fig 2. Constitutively active and dominantly negative forms of RapC.

(A) The amino acids sequences of the N-terminus of RapA and RapC. Asterisk indicates the locations of point-mutated amino acids for GFP-RapC^{G13V} and GFP-RapC^{S18N}. The sequences were confirmed by sequencing. (B) Confirmation of GFP-RapC-OE, GFP-RapC ^{G13V} and GFP-RapC^{S18N}.

GFP-fused RapC proteins were expressed in wild-type and the expression of the proteins was examined by western blotting using anti-GFP antibodies (expected size of GFP RapC, 57 kDa)

III-2. Opposite roles of RapA and RapC in cell morphology and adhesion.

To determine the antagonistic functions of cells overexpressing RapA and RapC, I measured and compared the areas of wild-type, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, GFP-RapC^{S18N}, and *rapC* null cells (Fig. 3A). Cells overexpressing RapA showed flat, spread-out morphological feature, and increased cell-substratum adhesion. Cells lacking RapC exhibited similar phenotypes in morphogenesis and cell attachment assay (Jeon et al., 2007; Park et al., 2018). GFP-RapA, GFP-RapA^{G14V}, GFP-RapC^{S18N}, and *rapC* null cells exhibited highly increased cell size. In contrast, GFP-RapA^{S19N}, GFP-RapC, and GFP-RapC^{G13V} cells showed decreased cell size, comparing with wild-type cells (Fig. 3B). GFP-RapA, GFP-RapA^{G14V}, and GFP-RapC^{S18N} were approximately 1.5-fold larger than wild-type cells, and *rapC* null cells were 2-fold larger than wild-type cells.

Next, to determine the opposite function of RapA and RapC in cell attachment, I analyzed cell adhesion by calculating the number of attached cells after shaking the plates for 1 h (Fig. 3D). GFP-RapA, GFP-RapA^{G14V}, GFP-RapC^{S18N}, and *rapC* null cells showed increased cell adhesion. However, GFP-RapA^{S19N}, GFP-RapC, and GFP-RapC^{G13V} cells showed decreased cell adhesion comparing to wild-type cells. Among cells having strong attachment to substrate, *rapC* null cells were the most adhesive, and cells expressing GFP-RapC showed the weakest adhesion.

These results suggest that both RapA and RapC play important roles in cell morphology and attachment of cell-substratum and they appeared to have the opposite functions in cell morphology and cell attachment to the substrate.

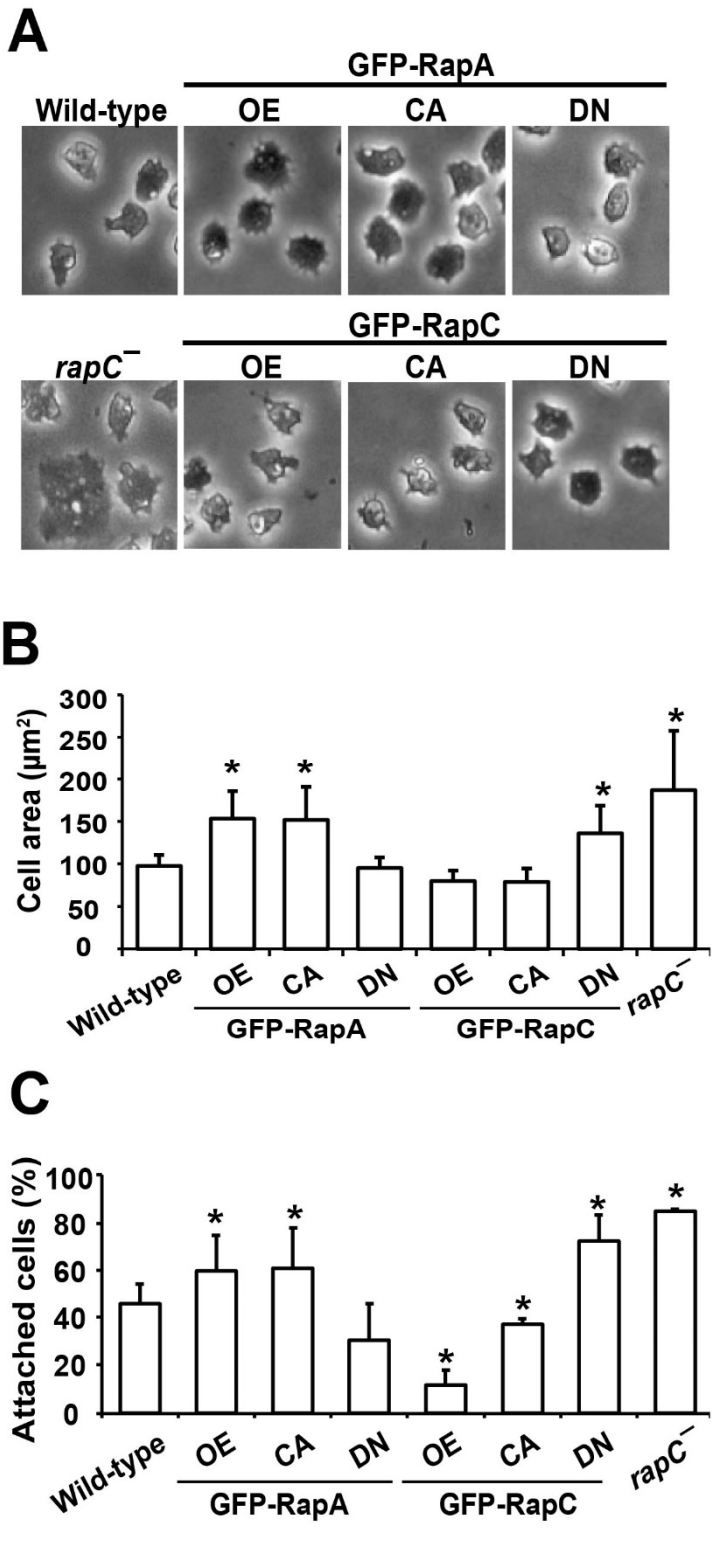


Fig 3. Cell morphology and adhesion of RapA and RapC cells.

(A) Morphology of the cells. Log-phase growing cells on the plates including wild-type, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, GFP-RapC^{S18N}, and *rapC* null cells were photographed using a phase-contrast microscope. (B) Measurement of cell size. The cell size was measured and graphed using ImageJ software. The values are the average ± SD of at least three independent experiments. (C) Cell-substratum attachment. The adhesion of the cells was measured as the ratio of attached cells after shaking to the total number of cells. Error bars represent SD. Statistically different from control (wild-type) at *p<0.05 by the student's t-test.

OE: Overexpression, CA: Constitutively active, DN: Dominantly negative

III-3. Unique role of RapC in cytokinesis.

To examine the functions of RapA and RapC in cytokinesis, I stained the cells, which were used previously in the experiments for cell morphology and adhesion, with Hoechst Dye and counted the nuclei of the cells. Wild-type cells, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, and GFP-RapC^{G13V} cells contained one or two nuclei averagely, whereas GFP-RapC^{S18N} or loss of *rapC* cells had three or four nuclei. Some of *rapC* null cells contained extremely larger number of nuclei over eight nuclei per one cell, while other cells had one or two (Fig. 4B). These data suggest that RapC is required for proper cytokinesis, and RapC has a unique function in cytokinesis, in contrast to RapA.

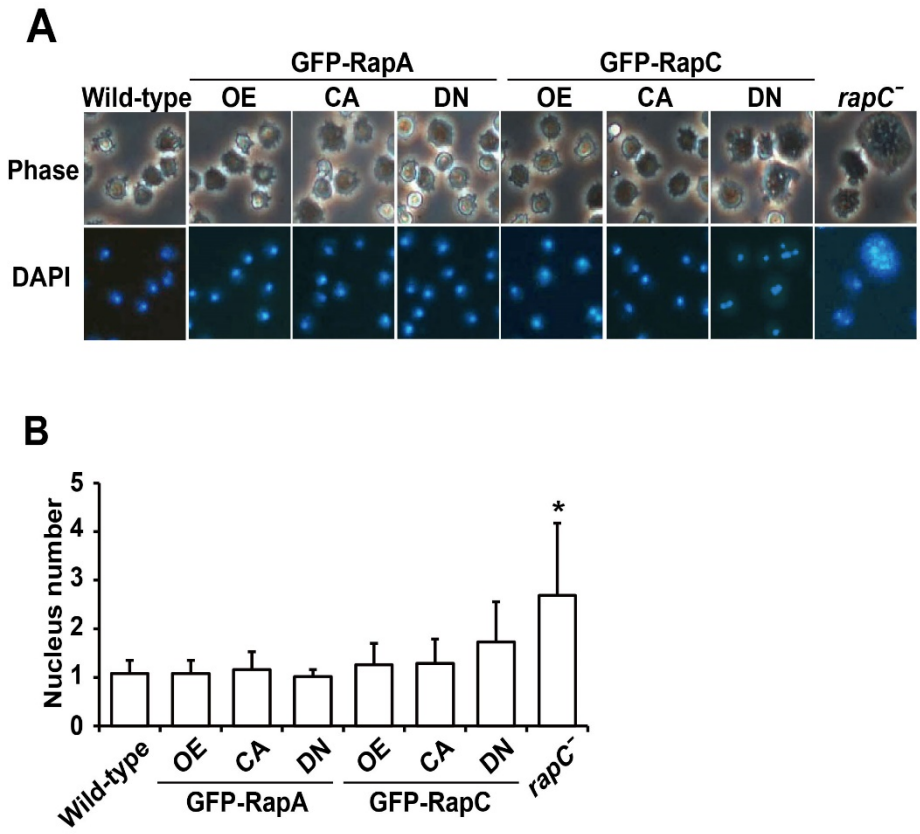
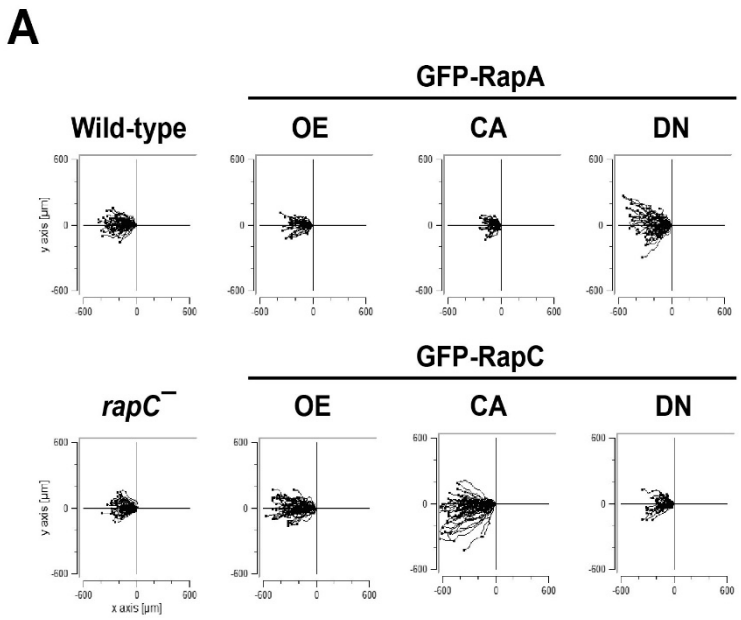


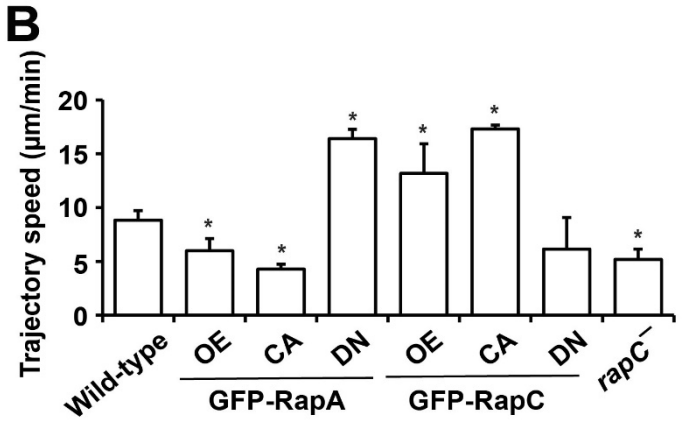
Fig 4. Cytokinesis of RapA and RapC cells.

(A) DAPI staining of RapA and RapC cells with Hoechst dye. Exponentially growing cells were stained with Hoechst dye and photographed with phase-contrast microscope. Representative phase-contrast (upper) and DAPI (lower) images are shown. Cells were attached on the plate for 20 mins and stained with 3 μg/mL concentration of Hoechst dye in PBS (pH 7.0). (B) Quantification of the number of nucleus. Error bars represent SD from three independent experiments. Statistically different from control (wild-type) at *p<0.05 by the student's t-test.

III-4. Opposite roles of RapA and RapC in chemotaxis.

To determine if RapA and RapC have opposite functions in cell motility as in cell morphology and cell attachment, I examined cell motility of the cells chemotaxing towards cAMP chemoattractants by using Dunn chemotaxis chamber (Fig. 5A). Aggregation competent cells were prepared by shaking and starving in Na/K phosphate buffer for 10 h. GFP-RapA, GFP-RapA^{G14V}, GFP-RapC^{S18N}, and *rapC* null cells having strong attachment to substrate migrate slowly to high concentrations of cAMP, comparing to wild-type cells. However, GFP-RapA^{S19N}, GFP-RapC, and GFP-RapC^{G13V} cells having weak attachment migrate fast to cAMP (Fig. 5B). There was no significant difference in the directionality of the cells moving toward high concentration cAMP (Fig. 5C). These results indicate that RapA and RapC are involved in controlling migration speed during chemotaxis in opposite ways.





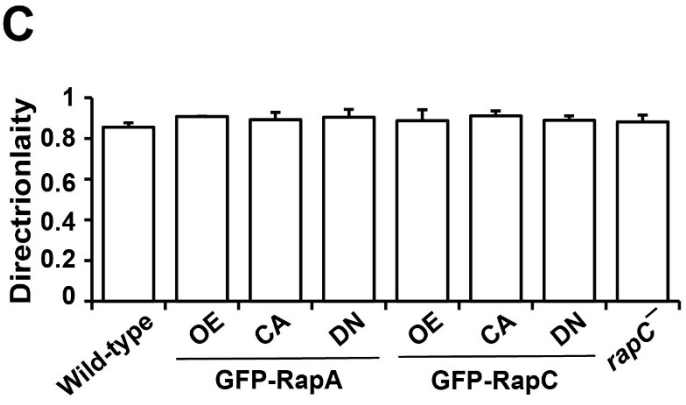


Fig 5. Cell migration of RapA and RapC cells.

(A) Trajectories of cells moving toward cAMP in Dunn chemotaxis chamber. Aggregation competent wild-type, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, GFP-RapC^{S18N}, and *rapC* null cells were placed in a Dunn chemotaxis chamber and the movement of cells from low cAMP concentration to high cAMP concentration were recorded using time lapse intervals for 1 hour at 1 min intervals. Every line in trajectories indicates the cells migrating toward cAMP. (B) Quantification of the migration speed in Dunn chemotaxis chamber. Trajectory speed indicates the entire distance which cells migrated toward cAMP divided by the time. (C) Directionality of the chemotaxing cells. Directionality evaluates how cells moved in straight line and the most straight forward moving cells have a value of 1. ImageJ software were used to analyze the recorded images. Statistically different from control (wild-type) at $*p<0.05$ by the student's *t*-test.

III-5. Unique role of RapC in multicellular development.

Dictyostelium cells secrete cAMP to surrounding cells during development. Cells aggregate to form mounds and finally form fruiting bodies (Chisholm and Firtel, 2004). To investigate possible roles of RapA and RapC during development, I examined the developmental phenotypes of the cells including wild-type, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, GFP-RapC^{S18N}, and *rapC* null cells (Fig. 6A). GFP-RapA, GFP-RapA^{G14V}, and GFP-RapC^{S18N} cells developed similar ways to wild-type cells, showing similar timing for development and developmental structures. GFP-RapA^{S19N}, GFP-RapC, and GFP-RapC^{G13V} cells showed slightly fast developmental process. There was no clear difference between RapA and RapC cells in each stage of development. However, *rapC* null cells exhibited unique developmental phenotypes, forming multiple tips from a single mound, suggesting that RapC plays a unique function in development and appear to be involved in the tip-forming stage during development.

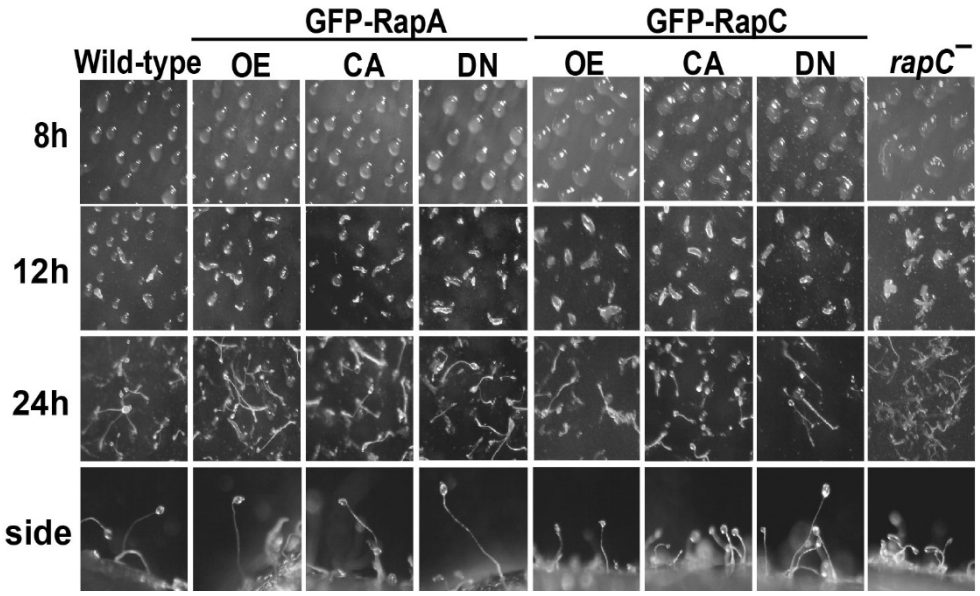


Fig 6. Multicellular development of RapA and RapC cells.

Representative images of development of wild-type, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, GFP-RapC^{S18N}, and *rapC* null cells. Log-phase cells were washed with Na/K phosphate buffer and developed on the non-nutrient plates. Developmental structures were photographed at the indicated times and representative images at the developmental stages are shown. The distinguishing stages of multicellular development at 8 hours (aggregation stage), 12 hours, 14 hours (slug and mound stage), and 24 hours (fruiting body stage) were shown.

III-6. Recovery of the phenotypes of *rapC* null cells in cell morphology and adhesion by RapA and RapC.

The previous experiments showed the opposite functions of RapA and RapC in the regulation of cell morphology, adhesion, and cell migration in chemotaxis. To determine whether RapA and RapC could complement the defects of *rapC* null cells in the cellular processes, I examined the morphological phenotype of wild-type cells, *rapC* null cells, *rapC* null cells expressing GFP-RapA, and *rapC* null cells expressing GFP-RapC (Fig. 7A). *rapC* null cells showed spread morphology as shown previously. The spread morphology of *rapC* null cells were rescued by expression of RapC but not by RapA. *rapC* null expressing RapA exhibited more spread morphology compared to *rapC* null cells (Fig. 7A and 7B). These results confirm the opposite roles of RapA and RapC in cell morphology; RapA promotes cell spreading but RapC restricts the cell size.

To determine if GFP-RapA or GFP-RapC could complement the defect of *rapC* null cells in cell adhesion, I examined cell adhesion of wild-type cells, *rapC* null cells and *rapC* null cells expressing GFP-RapA or RapC (Fig. 7D). *rapC* null cells and *rapC* null cells expressing GFP-RapA showed enhanced cell adhesion to substrate, whereas *rapC* null cells expressing GFP-RapC showed weak cell adhesion (Fig. 6E and Fig. 6F). These data indicate that the defects of *rapC* null cells in cell adhesion to substrate were complemented by expressing GFP-RapC, but not GFP-RapA.

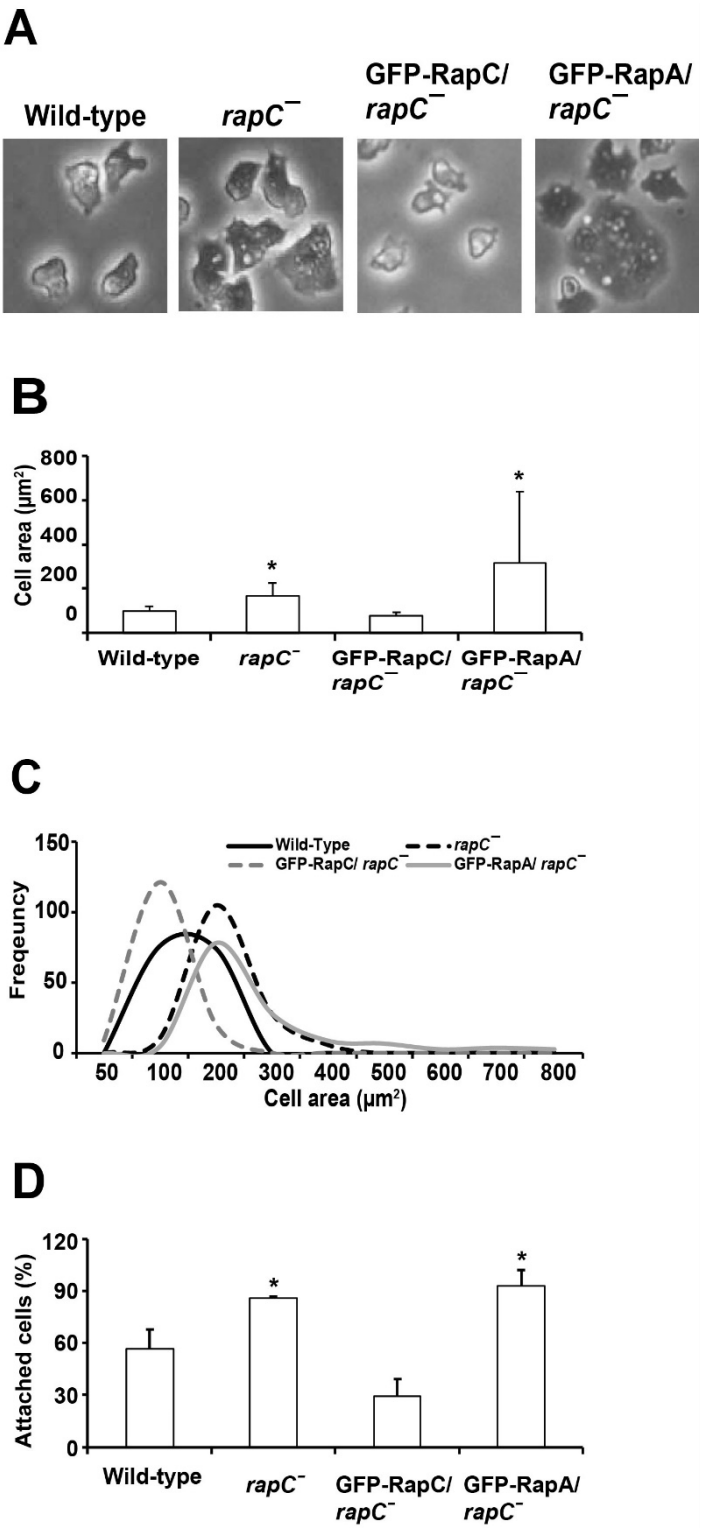


Fig 7. Cell morphology and adhesion of *rapC* null cells by RapA and RapC.

(A) Cell morphology of vegetative wild-type, *rapC* null, GFP-RapC/ *rapC* null, and GFP-RapA/ *rapC* null cells. (B) Quantification of the cell area. The cells were photographed and the cell sizes were measured using ImageJ software. (C) Frequency of cell areas. The frequency of different cell sizes was counted and graphed. (D) Cell adhesion. The adhesion of the cells to the substrate was presented as the ratio of attached cells after shaking off the detached cells from the plates to the total number of cells. Error bars represent SD from three independent experiments. Statistically different from control (wild-type) at $*p<0.05$ by the student's *t*-test.

III-7. Recovery of the cytokinesis defects of *rapC* null cells by RapA and RapC.

rapC null cells are abnormally enlarged and multinucleated resulting from severe defects in cytokinesis (Park et al., 2018). To determine if the multinucleated phenotypes of *rapC* null cells are recovered by RapA and RapC, I examined the number of nuclei of the cells by staining the cells with Hoechst dye (Fig. 8). The majorities of *rapC* null cells and *rapC* null cells expressing GFP-RapA contained three or more nuclei per one cell, while *rapC* null cells expressing GFP-RapC had one or two nuclei (Fig. 8B). These data indicate that the phenotypes of *rapC* null cells in cytokinesis were rescued by RapC but not by RapA.

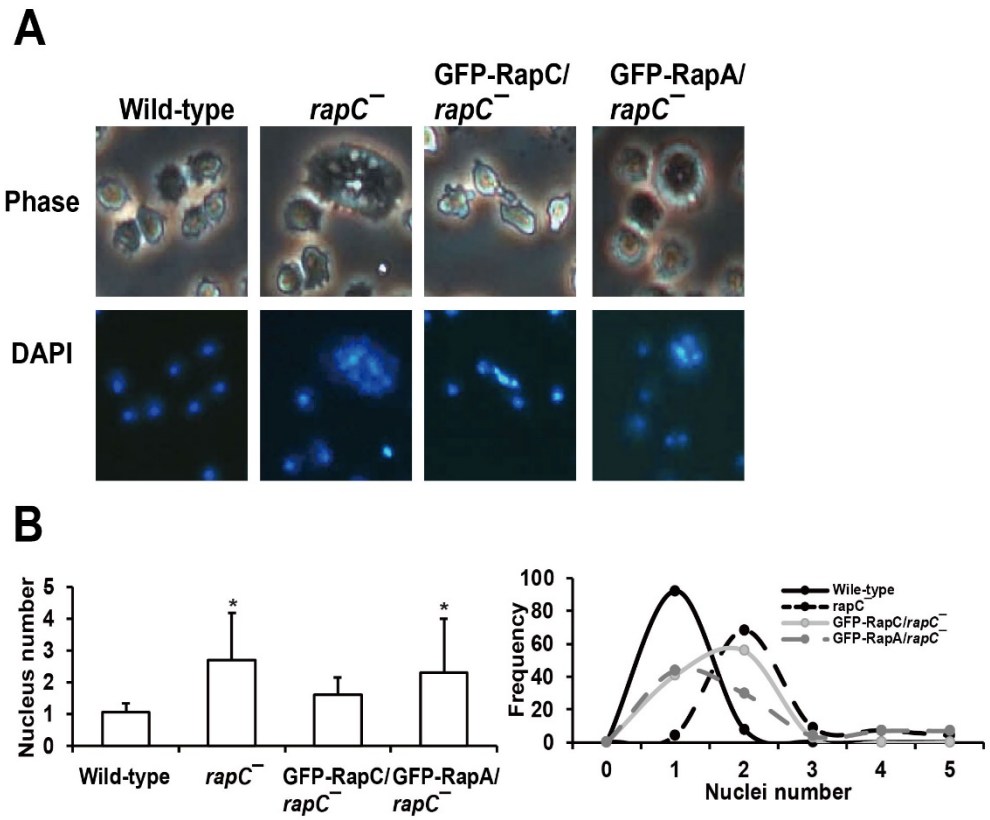


Fig 8. Cytokinesis of *rapC* null cells by RapA and RapC.

(A) Phase-contrast images and staining of the nucleus of the cells. Wild-type, *rapC* null cells, GFP-RapC/ *rapC* null cells, and GFP-RapA/ *rapC* null cells were plated and stained with Hoechst dye. Phase-contrast images and DAPI images were shown. (B) Quantification of the number of nucleus in the cells. Error bars represent SD from three independent experiments. Statistically different from control (wild-type) at **p*<0.05 by the student's *t*-test.

III-8. Recovery of the phenotypes of *rapC* null cells in cell migration by RapA and RapC.

rapC null cells and cells overexpressing RapA have a defect in the control of migration speed during chemotaxis towards cAMP chemoattractants (Jeon et al., 2007; Park et al., 2018). To determine if the expression of RapA and RapC could complement the slow cell motility during chemotaxis of *rapC* null cells, I investigated chemotaxis of the cells in using a Dunn chemotaxis chamber (Fig. 9A). The migration speed of *rapC* null cells and *rapC* null cells expressing GFP-RapA was slower than wild-type cells, whereas *rapC* null cells expressing GFP-RapC had the same migration speed as wild-type cells (Fig. 9B). There was no significant difference in directionality among the cells (Fig. 9C). These results indicate that the phenotypes of *rapC* null cells in cell migration was complemented by RapC but not by RapA. In addition, the results suggest that RapA decreases the migration speed while RapC increases.

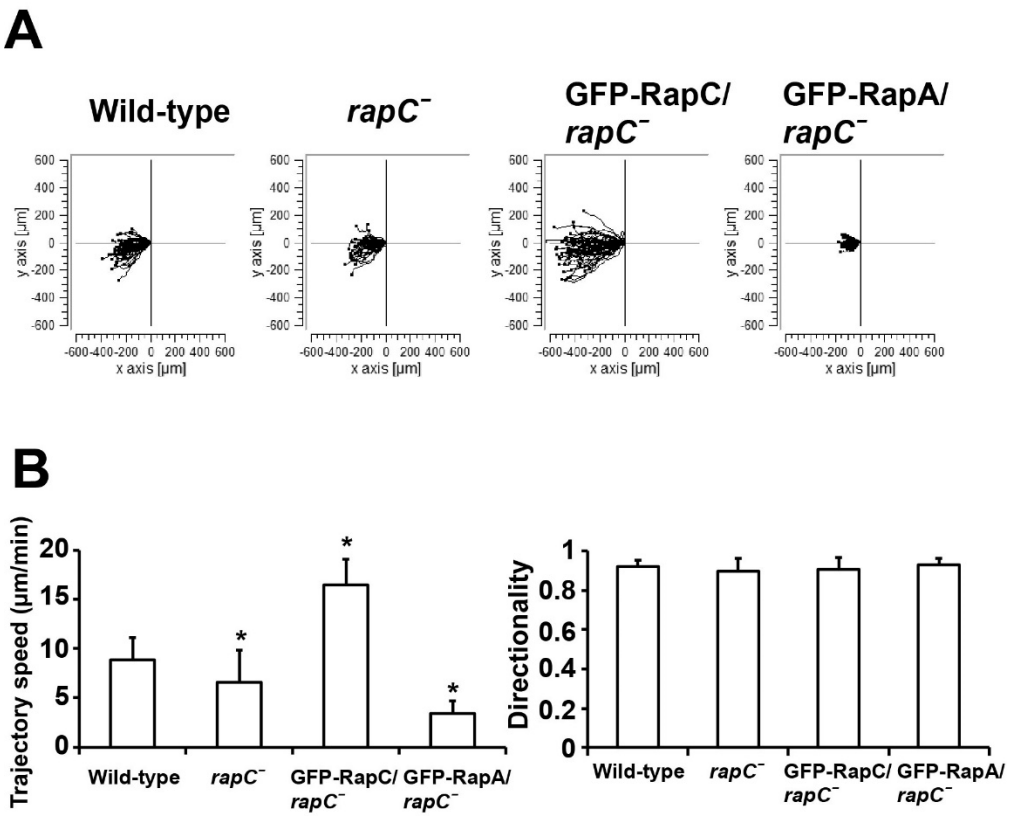


Fig 9. Chemotaxis of *rapC* null cells by RapA and RapC.

(A) Trajectories of cells moving toward 1 μM cAMP in Dunn chemotaxis chamber. Aggregation competent wild-type cells, *rapC* null cells, *rapC* null cells expressing GFP-RapC cells, and *rapC* null cells expressing GFP-RapA cells were placed in a Dunn chemotaxis chamber. The movement of cells from low cAMP concentration to high cAMP concentration were recorded using time lapse intervals for 1 hour at 1 min intervals. Every line in trajectories indicates the cells migrating toward cAMP. (B) Quantification of the migration speed in Dunn chemotaxis chamber. Trajectory speed indicates the entire distance which cells migrated toward cAMP divided by the time. (C) Quantification of directionality of the cells in chemotaxis. Directionality evaluates how cells moved in straight line and the most straight forward moving cells have a value of 1. ImageJ software were used to analyze the recorded images. Error bars represent SD from three independent experiments. Statistically different from control (wild-type) at $*p<0.05$ by the student's *t*-test.

III-9. Recovery of the developmental phenotypes of *rapC* null cells by RapA and RapC.

To confirm that RapC has a unique function in development, I determined if the developmental phenotypes of *rapC* null cells were complemented by expression of RapA and RapC (Fig. 10). The cells were developed on non-nutrient agar plates and photographed at the indicated times. Wild-type cells showed aggregation of the cells at 8 h after development, tip-formation and slug stage at 12 h, and then finally formation of fruiting bodies at 24 h. *rapC* null cells exhibited severe defects in development, forming multiple tips from a single mound and delayed formation of fruiting bodies. These phenotypes of *rapC* null cells were complemented by expression of RapC but not by RapA. These results confirm that RapC plays an important role in the formation of tip from an aggregate.

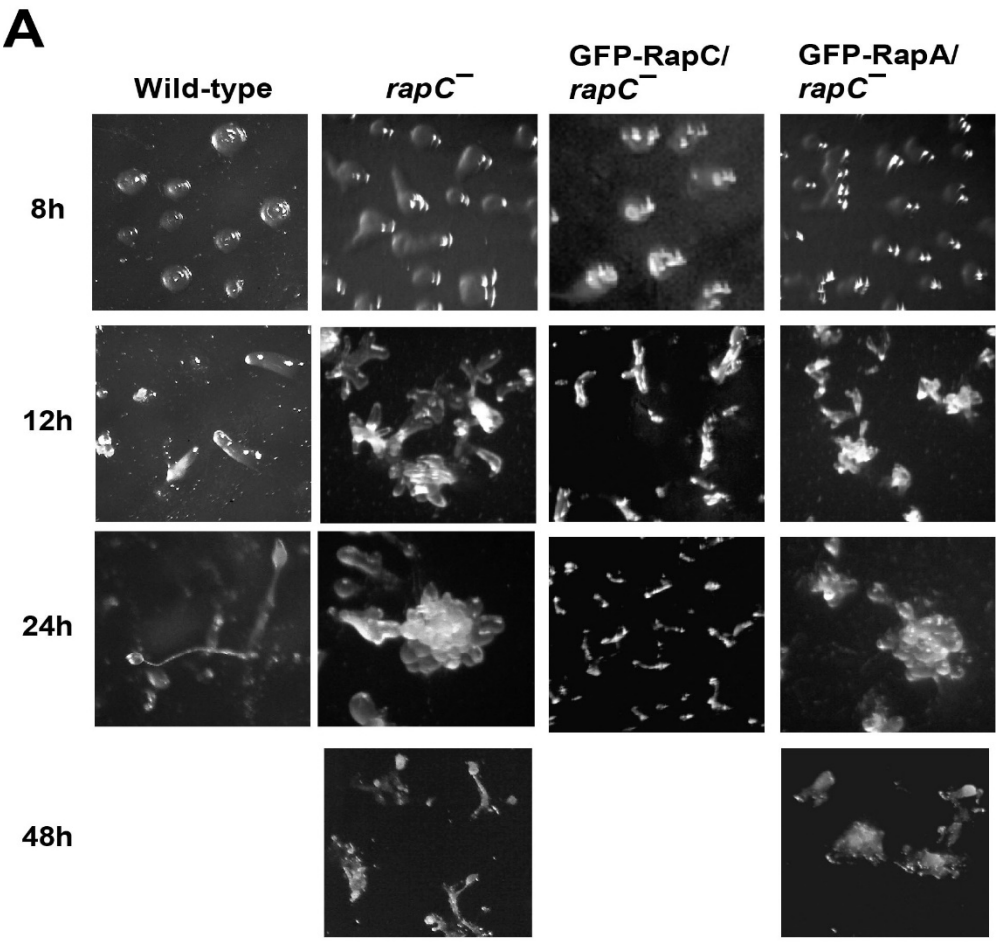


Fig 10. Development of *rapC* null cells by RapA and RapC.

(A) Development of wild-type cells, *rapC* null cells, *rapC* null cells expressing GFP-RapC, and *rapC* null cells expressing RapA cells. Log-phase growing cells were developed on non-nutrient agar plates. Photographs were taken at the developmental stages of development, 8 h for aggregation stage, 12 h for slug and mound stage, and 24-48 h for fruiting body formation stage. (B) Side view of fruiting bodies at 24 h development.

III-10. Subcellular localization of RapA and RapC.

I investigated the subcellular localization of RapA and RapC using GFP tagged RapA and RapC (Fig. 11). The fluorescence images were captured by using NIS-elements software. GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, and GFP-RapC^{S18N} were found at the plasma membrane and also were observed in the intracellular structures.

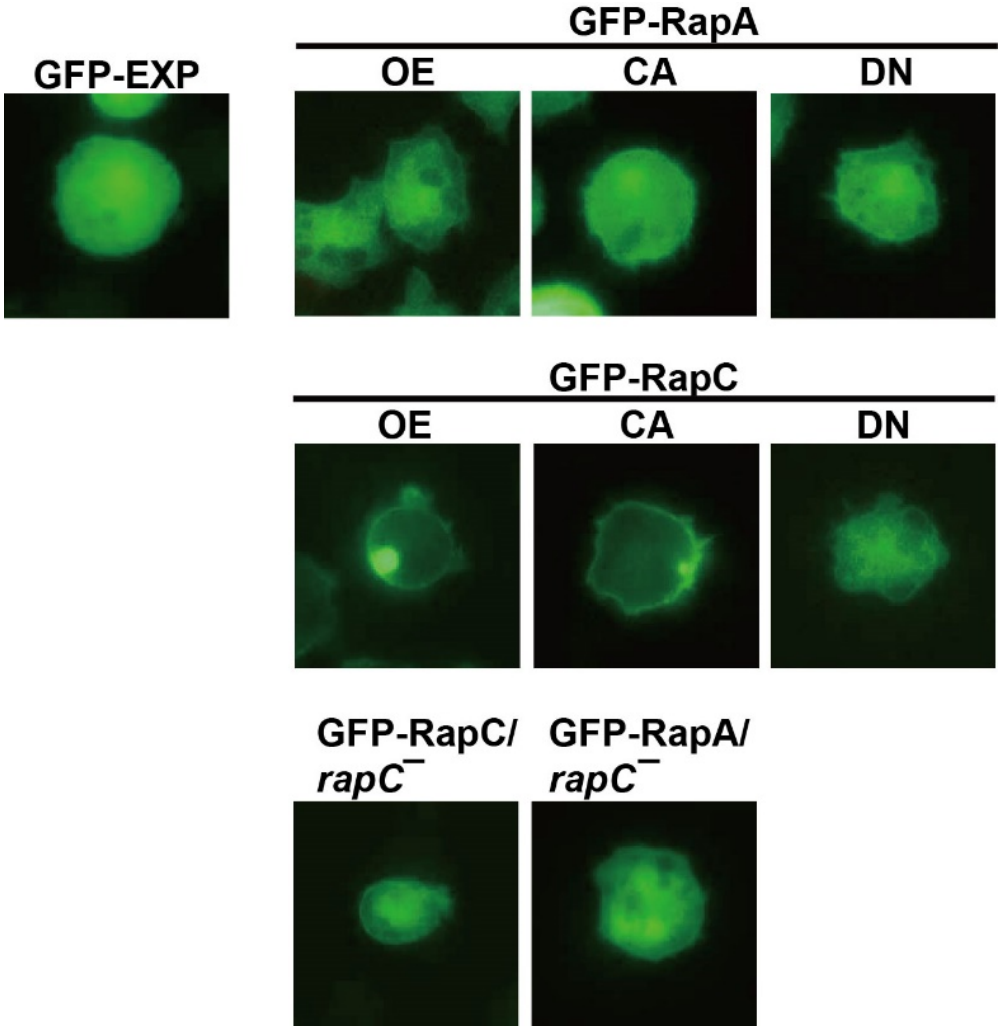


Fig 11. Subcellular localization of GFP-RapA and GFP-RapC.

Exponentially growing wild-type cells expressing GFP-exp, GFP-RapA, GFP-RapA^{G14V}, GFP-RapA^{S19N}, GFP-RapC, GFP-RapC^{G13V}, and GFP-RapC^{S18N} were placed on the coverslip and fluorescence images of the cells were capture by using NIS-elements software. *rapC* null cells expressing GFP-RapC or GFP-RapA were also photographed.

IV. Discussion

The small GTPase RapA is the most extensively studied Rap protein in *Dictyostelium*. RapA is a key regulator of cell survival, cell attachment to substrate, and cell migration towards chemoattractant. RapC is the closest homolog to RapA. Recent papers showed that RapA and RapC have the opposite roles in diverse cellular processes, such as cell spreading, cell attachment, and motility (Jeon et al., 2007; Park et al., 2018).

In this study, I compared the phenotypes of the cells overexpressing wild-type (OE), constitutively active (CA), and dominantly negative (DN) forms of RapA and RapC in various biological processes including morphogenesis, cell attachment, and cell migration. Additionally, I compared whether the phenotypes of *rapC* null cells could be complemented by RapA or RapC. Cells expressing GFP-RapA and GFP-RapA^{G14V} showed spread morphology and strong cell-substrate attachment, while cells expressing dominantly negative form of RapA (GFP-RapA^{S19N}) showed shrank cell area and weak cell adhesion. In contrast, cells expressing wild-type form or constitutively active form of RapC (GFP-RapC and GFP-RapC^{G13V}) showed decreased cell area and cell adhesion, whereas *rapC* null cells and cells expressing dominantly negative form of RapC (GFP-RapC^{S18N}) showed flat and spread and strong cell adhesion. These results indicate that RapA and RapC have antagonistic effects on cell morphology and cell adhesion, RapA and RapC promoting and preventing cell spreading and cell adhesion, respectively. The defects of *rapC* null cells in regulation of cell spreading and cell adhesion were complemented by expression of RapC, but not RapA, supporting the opposite function of RapA and RapC in the control of cell morphology and adhesion.

It appears that RapC has a unique function in cytokinesis. All cells except *rapC* null cells and cells expressing RapC-DN contained one nucleus as shown in wild-type cells. *rapC* null cells were multinucleated and the phenotypes were complemented by

expression of wild-type form of RapC but not RapA.

Cell migration is involved in many biological and pathological processes, such as embryonic development, wound healing, inflammatory responses, and tumor metastasis (Jin et al., 2009; Lee and Jeon, 2012; Ridley et al., 2003). The migration speed of RapA-OE, RapA-CA, and RapC-DN cells were slower than wild-type cells, while RapA-DN, RapC-OE, and RapC-CA cells were fast compared to wild-type cells. These data reveal that activated RapA decreases while RapC increases the migration speed.

Contrary to the opposite functions of RapA and RapC in cell migration, RapC appears to have a unique function in multicellular development. *rapC* null cells were defective in development, forming multiple tips from a single mound. These phenotypes were complemented by only RapC but not RapA.

Among the Ras family, Rap1 has been extensively studied because of its critical roles in cellular processes including integrin-mediated cell adhesion, cadherin-mediated cell-cell adhesions, formation of cell polarity, cell survival, and cell spreading (Lee and Jeon, 2012). Rap proteins are consisted of Rap1 and Rap2 in mammalian cells, and they share 60% identities (Carvalho et al., 2019). Despite of their high homology, reverse regulatory roles of Rap1 and Rap2 on endothelial barrier resistance in HUVEC has been recently reported. The activation of Rap1 raised barrier resistance, while the activation of Rap2 decreased the effect (Pannekoek et al., 2013). Similar results were observed in our study. Although RapA and RapC showed high homology (51%), the antagonistic effects of RapA and RapC in morphogenesis, cell adhesion, and cell migration were shown. RapC has an additional stretch of amino acids at the C-terminus of RapC. The C-terminus of RapC might contribute to the opposite functions of RapC.

Opposite functions of RapA and RapC in morphogenesis, cell adhesion, cell motility, and development were summarized in Table.2. The complemental phenotypes of *rapC* null cells by RapA and RapC in cell morphology, cell attachment, cell migration, and

development were presented in Table.3.

Table 2. Summary of the opposite functions of RapA and RapC.

	RapA OE	RapC OE	*rapC*$^{-}$
Cell size	**Enlarged**	**Shrank**	**Enlarged**
Cell adhesion	**Strong**	**Weak**	**Strong**
Cytokinesis	**Normal**	**Normal**	**Multinucleated**
Motility	**Slow**	**Fast**	**Slow**
Development	**Delayed (single tip)**	**Accelerated (single tip)**	**Delayed (multiple tips)**

Table 3. Summary of the phenotypes of *rapC* null cells by RapA and RapC.

	rapC^{-} cells	GFP-RapC/ *rapC*$^{-}$ cells	GFP-RapA/ *rapC*$^{-}$ cells
Cell size	**Enlarged**	**Shrank**	**Enlarged**
Cell adhesion	**Strong**	**Weak**	**Strong**
Cytokinesis	**Multinucleated**	**Normal**	**Multinucleated**
Motility	**Slow**	**Fast**	**Slow**
Development	**Delayed (multiple tips)**	**Accelerated (single tip)**	**Delayed (multiple tips)**

Part II. Roles of RasZ protein in *Dictyostelium* cell migration and development

I. INTRODUCTION

Chemotaxis is required for various biological processes such as immune responses of lymphocytes and leukocytes, and neuronal patterning in the development of neuronal system. Abnormal cell migration results in the diverse human diseases including inflammatory diseases and metastatic cancer (Charest et al., 2010; Jin et al., 2009; Jin et al., 2008). Understanding the basic mechanisms underlying cell motility could facilitate the ability of effective approaches for treating disease, preparation of synthetic tissues, and cellular transplantation (Ridley et al., 2003). Despite of the importance of the cell migration, little is known about the molecular processes (Jin et al., 2009). Cell migration is mediated by a series of signaling molecules including G-protein coupled receptors (GPCRs), Ras proteins, PI3Kinase, and F-actin regulating proteins. For cells to directionally migrate, coordinated assembly and disassembly of cytoskeleton at the leading edge and the posterior of the migrating cells. At the leading edge of the migrating cells, F-actin is polymerized to lead the protrusion and forward movement. At the posterior and lateral sides of the migrating cells, myosin II is assembled to furnish cortical tension and retract the posterior (Jeon et al., 2007).

Free-living social amoeba, *Dictyostelium discoideum,* is a good model system for understanding chemotaxis, and Ras signaling pathways. (Eichinger et al., 2005; Lee and Jeon, 2012). The migration of the cells in chemotaxis is initiated by binding of the chemoattractants to GPCRs. Four cAMP receptors (cARs) have been identified in

Dictyostelium: cAR1 is the most essential receptors among 4 cARs and showed high sensitivity to cAMP chemoattractant, cAR2-4 showed low sensitivity to cAMP, but are important for the early multicellular development. Ras proteins in human oncogene have been the subject of concentrated research. *Dictyostelium* has 19 Ras GTPase subfamily proteins; 15 Ras, 3 Rap, and one Rheb related protein (Kortholt and van Haastert, 2008).

Ras proteins cycle between GTP-bound active form factors (GEFs) and GDP-bound inactive form, which are regulated by guanine nucleotide exchange factors (GEFs) and by GTPase-activating proteins (GAPs) (Park et al., 2018). Rap1 is the closest homolog of Ras GTPase (Bos and Zwartkruis, 1999). Rap1 is rapidly activated at ~6s in response to chemoattractant stimulation and activated Rap1 localizes at the leading edge of cells migrating towards chemoattractants. The activated Rap1 at the leading edge leads to cell attachment and contribute to cell polarity formation by regulating Myosin II assembly and disassembly though the Rap1/ Phg2 signaling pathway (Jeon et al., 2007). Although Ras proteins were investigated over than 30 years, the functions of Ras family proteins have not been examined yet. In this study, I investigated the functions of RasZ using *rasZ* null cells and *rasZ* null cells expressing RasZ in the regulation of cell morphology, cell adhesion, migration, and development.

II. MATERIALS AND METHODS

II-1. Strains and cell culture

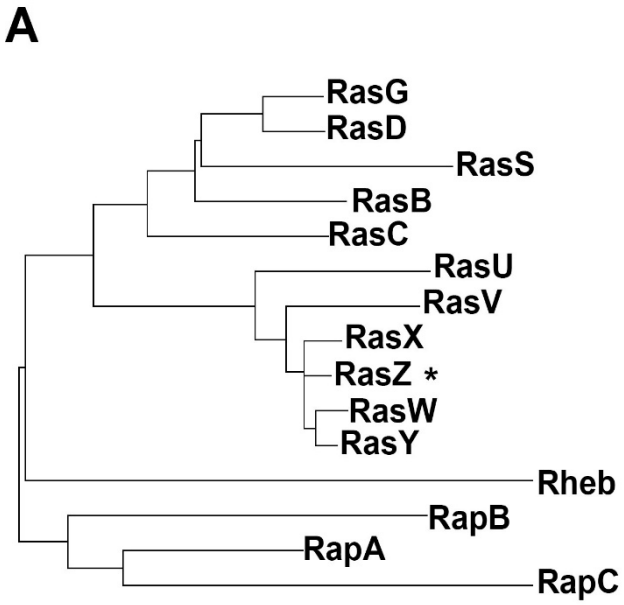
Exponentially growing *Dictyostelium discoideum* cells were cultured in the culture plates with HL5 medium at 22 °C (Nellen et al., 1984). 6 mL 100X antibiotic-antimycotic solution and 16 mL 50 % glucose were added in HL5. 10 μg/mL G418 was supplemented to the cells expressing green fluorescent protein (GFP) RasZ. *rasZ* null cells (DBS0236874) were obtained from NBRP Nenkin (Japan) (Muramoto et al., 2005). The coding sequence of *rasZ* cDNA was prepared by RT-PCR and was cloned into the *Eco*RI – *Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment.

III. RESULTS

III-1. Phylogenetic analysis of RasZ.

There are 15 Ras proteins in Dictyostelium discoideum: 11 Ras (RasB, RasC, RasD, RasG, RasS, RasU, RasV, RasW, RasX, RasY, and RasZ), 3 Rap (RapA, RapB, and RapC), and one Rheb like protein (Fig. 12A).

To characterize the functions of RasZ in *Dictyostelium*, I performed computer analysis. *Dictyostelium* RasZ (dictyBase ID: DDB_G0270140) is composed of 214 amino acids and has a RAS doamin at the N-terminal region which is alike with RasX and RasY (Fig. 12B). Among the Ras family proteins, RasZ formed a group with RasX, RasY, and RasW, showing high homology of 90.23 %, 89.1 %, and 82 %, respectively (Fig. 12B).



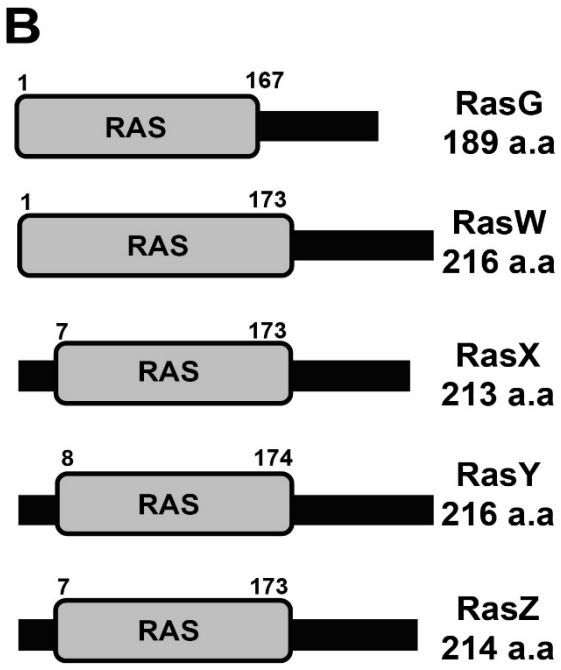


Fig 12. Phylogenetic tree and domain structures of Ras family proteins.

(A) Phylogenetic tree analysis of RAS proteins in *Dictyostelium*: RapA(dictyBase ID: DDB_G0216229), RapB(dictyBase ID: DDB_G0272857), RapC(dictyBase ID: DDB_G0272857DDB_G0270340), RasB(dictyBase ID: DDB_G0292998), RasC(dictyBase ID: DDB_G0281385), RasD(dictyBase ID: DDB_G0292996), RasG(dictyBase ID: DDB_G0293434), RasS(dictyBase ID: DDB_G0283537), RasU(dictyBase ID: DDB_G0270138), RasV(dictyBase ID: DDB_G0270736), RasW(dictyBase ID: DDB_G0270122), RasX(dictyBase ID: DDB_G0270124), RasY(dictyBase ID: DDB_G0270126), RasZ(dictyBase ID: DDB_G0270140), cpras1(dictyBase ID: DDB_G0277381), Rheb(dictyBase ID: DDB_G0277041), rsmA(dictyBase ID: DDB_G0283547), rsmB(dictyBase ID: DDB_G0281253), and DDB G0280437. The phylogenetic tree was drawn by MEGA7 to see the homolog among RAS domains. (B) Domain structures of RasZ and other Ras proteins. All of those Ras proteins have RAS domain at the N-terminus.

III-2. RasZ is involved in the regulation of cell morphology and adhesion.

To investigate the functions of RasZ, I compared the phenotypes of wild-type cells, *rasZ* null cells, and *rasZ* null cells expressing RasZ. *rasZ* null cells were rounded and smaller than wild-type cells (Fig. 13A). Quantification of the cell size was measured using NIS-element. The average cell area of the *rasZ* null cells was nearly half-fold smaller than wild-type cells (Fig. 13B). The morphological phenotypes of *rasZ* null cells was complemented by expressing RasZ. These data indicate that RasZ is involved in the regulation of cell morphology. Next, I examined cell adhesion of the wild-type cells, *rasZ* null cells, and *rasZ* null cells expressing RasZ by quantifing the fraction of cells that attached to the plates during agitation. *rasZ* null cells showed weak adhesion comparing to wild-type cells, and the defect of cell adhesion was complemented by expression of GFP-RasZ (Fig. 13C). These data suggest that RasZ is required for cell adheison.

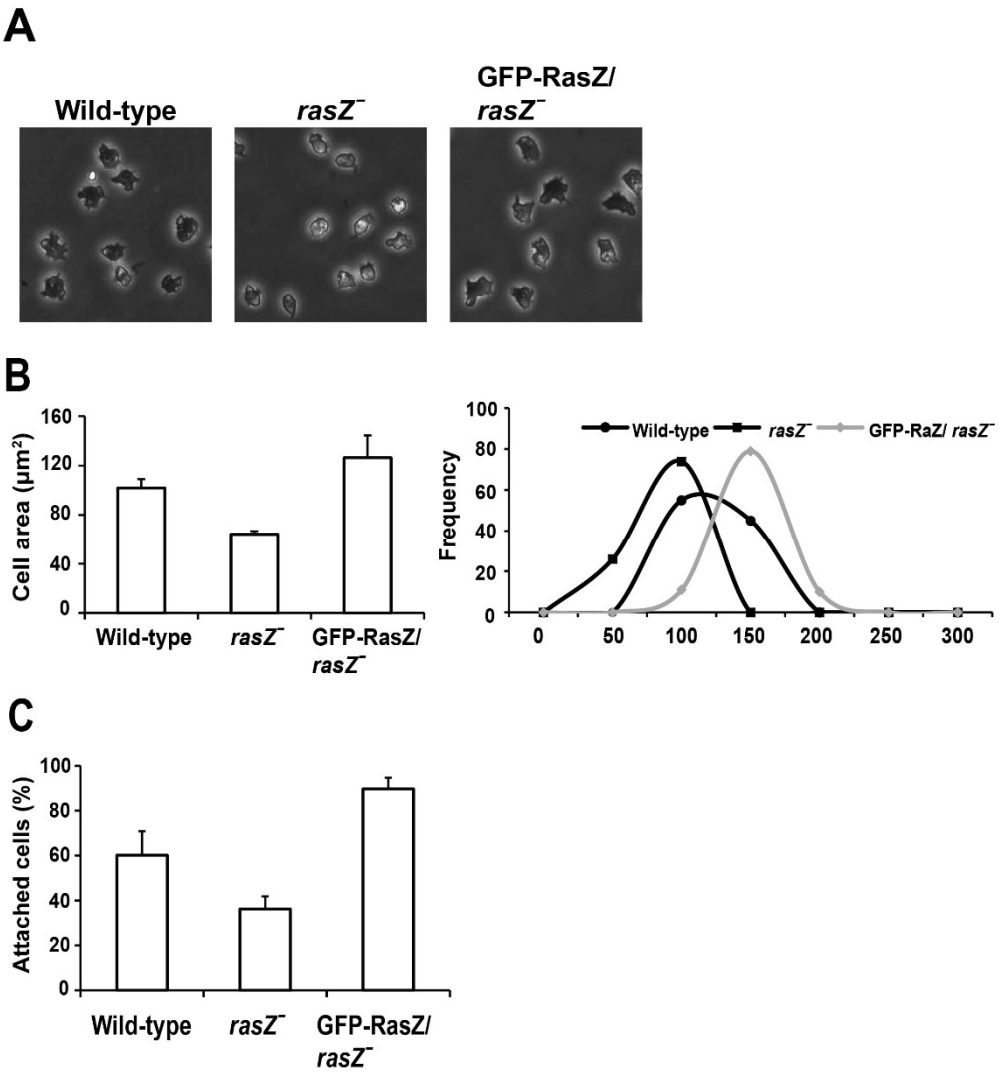


Fig 13. Cell morphology and cell adhesion of *rasZ* null cells and RasZ overexpressing cells.

(A) Morphology of wild-type cells, *rasZ* null cells, and GFP-RasZ expressing cells. The cells were photographed by NIS-elements. (B) Quantification of the cell morphology. The histogram indicates areas of cells (left) and the frequency of the cell area is shown (right). (C) Cell-substrate adhesion. Attachment of the cells was calculated as a percentage of attached cells to total cells.

III-3. RasZ is required for cell migration and development.

The previous results indicated that RasZ is involved in reorganizing the cell morphology and cell attachment. RapA is the most studied protein in *Dictyostelium* and the cells overexpressing RapA showed spread out and flat morphology, strong cell attachment with substrate, and decreased cell migration speed during chemotaxis (Jeon et al., 2007). *rap*C null cells and *rapgap*9 null cells showed similar results in those results (Mun et al., 2014; Park et al., 2018). These data suggest the possibility of requirement of RasZ in cell motility and multicellular developmental processes in *Dictyostelium*. Therefore, I investigated cell migration of *rasZ* null cells to chemotax up a cAMP gradient using Image J software and NIS-element software. There was no significant difference in directionality. However, the trajectory speed of *rasZ* null cells was slightly increased compared to wild-type cells (Fig. 14A and 14B). These data indicate that RasZ is involved in cell migration but not directionality during chemotaxis.

Next, I compared developmental phenotypes of wild-type cells, *rasZ* null cells, and *rasZ* null cells expressing RasZ on the non-nutrient agar plates. Under the non-nutrient environment, *Dictyostelium* aggregates by secreting cAMP to surrounding cells to establish the formation of the multicellular development. *rasZ* null cells formed normally mound at 8 hours (aggregation stage), and the size of mound was similar to wild-type cells (Fig. 15A). Interestingly, the spore size of *rasZ* null cells was approximately 2-fold smaller than wild-type cells and the stalk length was 2-fold longer than wild-type cells (Fig. 15). These results indicate RasZ is involved in the multicellular development and plays an important role in the regulation of the size of spores and stalks.

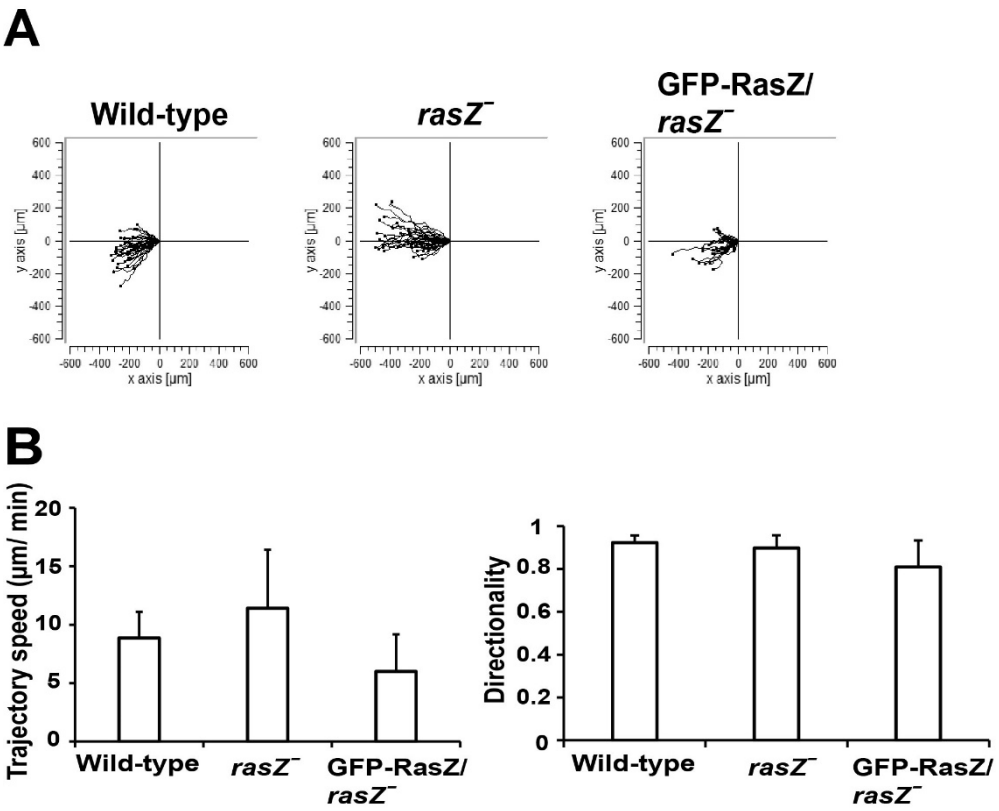


Fig 14. Cells migration of *rasZ* null cells and RasZ overexpressing cells.

A) Trajectories of chemotaxing cells toward cAMP in Dunn chemotaxis chamber. Aggregation competent wild-type cells, *rasZ* null cells, and RasZ overexpressing cells were prepared in the Dunn chemotaxis chamber, and the motility of cells to high cAMP concentration were recorded by time lapse photography for 1 h at 1 min intervals. (Each line in trajectories represents the cells moving toward cAMP. (B) Measurement of the migration speed and directionality of the chemotaxing cells. Directionality indicates how cells migrated in straight line and the most straight forward moving cells have a value of 1 (left). Trajectory speed represents total distance which cells moved toward chemoattractant, cAMP, divided by the time (right). ImageJ software waa used to analyze the recorded images

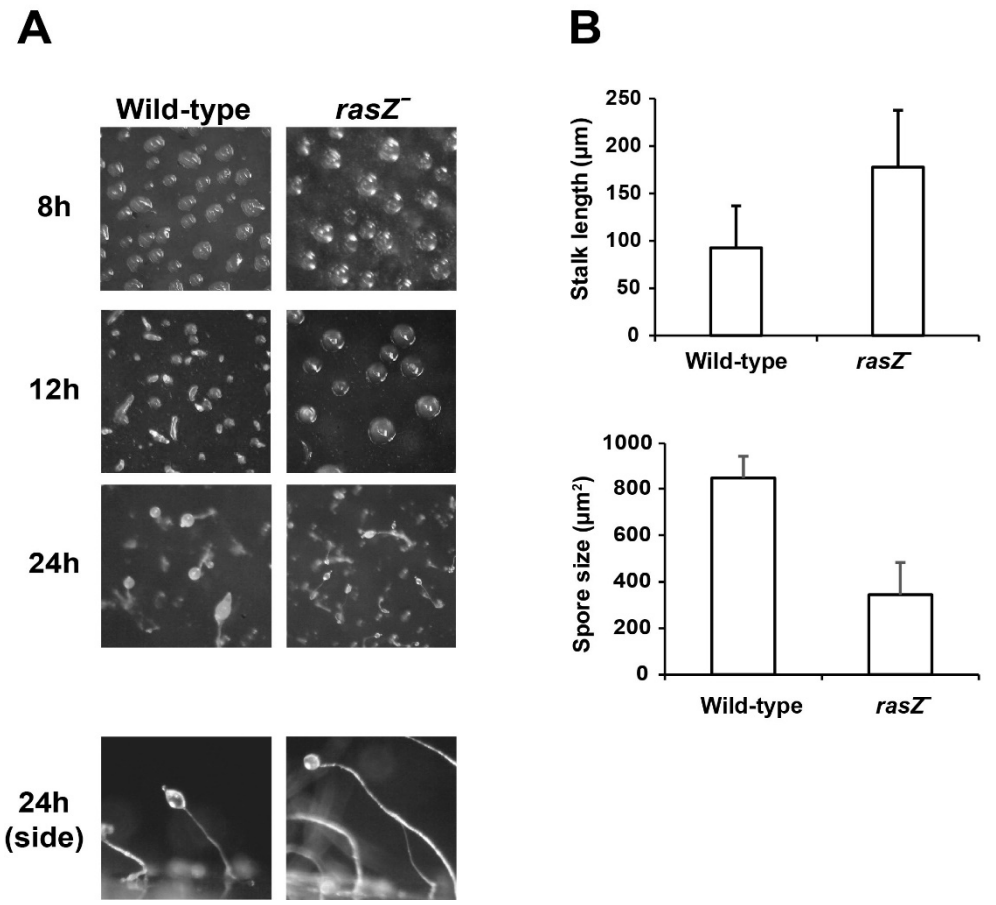


Fig 15. Development of *rasZ* null cells.

(A) Representative images of developmental structures of wild-type cells and *rasZ* null cells. Log-phase grown cells were washed with 12 mM Na/K phosphate buffer (pH 6.1) and placed on the non-nutrient agar plates. Photographs were captured at the indicated times. (B) Quantification of the size of the spore and stalks. The spores and stalks of fruiting bodies after 24 h development were measured using ImageJ software.

IV. Discussion

Ras small GTPase proteins are involved in various cellular signaling pathways including cell migration, cell survival, and cell cycle progression (Rajalingam et al., 2007). Among Ras family protein, Rap1 is the most widely studied to date because of its importance, including cell adhesion to substrate, cell migration through regulation of the arrangement of cytoskeleton, and cell polarity formation in *Dictyostelium* (Jeon et al., 2007). Although the essential roles of Ras proteins in cellular processes, little is known about the functions of them.

In this study, I demonstrated the functions of RasZ in *Dictyostelium*. In computer analysis, RasZ belongs to a group with other Ras proteins such as RasW, RasX, RasY, and RasZ in phylogenetic tree. RAS domain of RasZ showed 90.23 %, 89.1 %, and 82 % sequence similarities with RasX, RasY, and RasW, respectively. The results show that RasZ plays a negative role for cell spreading and cell-substrate adhesion, and cell migration. *rasZ* null cells showed decreased cell area and reduced cell-substrate attachment compared to wild-type cells. *rasZ* null cells expressing RasZ (GFP-RasZ/ *rasZ* null cells) rescued the defects of rasZ null cells in spreading cell shape and cell attachment to substrate. In addition, *rasZ* null cells had increased cell migration speed, while *rasZ* null cells expressing RasZ displayed normal migration speed similar to that of wild-type cells. During multicellular development, *rasZ* null cells showed decreased spore size and stretched stalk length.

Cell migration is a coordinated process of F-actin-related protrusions at the leading edge and myosin-related contraction of the rear of a cell. Ras proteins are involved in various biological processes including cell adhesion, cell migration, and cell survival in *Dictyostelium*. I investigated the roles of RasZ. The results indicate that RasZ is involved

in cell morphogenesis, cell-substrate adhesion, and cell migration. This study will provide further insight into understanding Ras signaling pathways.

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처음 연구실 생활을 했을 때 많이 낯설고 혹시 내 실수로 인해 다른 사람에게 피해가 가지 않을까 걱정을 해 두려웠지만 옆에서 응원과 격려를 아끼지 않고 실험실 분위기를 밝게 만들어 주었던 동주 오빠, 문제가 있을 때마다 달려와 자기일 인 것 같이 함께 열심히 고민해주던 밥 친구 원범 오빠, 대학교 입학부터 대학원 졸업까지 고민을 같이 들어주고 용기와 격려를 아끼지 않아 나를 항상 다잡아줬던 지성이, 항상 장난을 잘 받아주던 상철이

모두 감사해요. 각자 생활이 바빠서 자주 보지는 못했지만 힘들 때마다 위로와 응원으로 내 대학원 생활동안 활력소를 담당해준 은경이, 수현이, 내 스케줄 때문에 자주 못 만났지만 이해해주고 동해에서 항상 밝게 맞아주던 성우, 주원이, 정원이, 혜원이 모두 고마워, 우리 우정 오래가자... 내가 진짜 잘할게!!! 실험실 생활을 하며 많은 도움과 조언을 해주신 김미은 박사님, 항상 응원해주시는 성훈이 오빠, 주원이 오빠, 영빈이 오빠, 요한이 오빠, 수민이 오빠, 항상 웃는 얼굴로 저를 맞아주고 힘들 때마다 많은 얘기를 통해 우울했던 저를 즐겁게 만들어 주신 진솔이 언니, 각자 분야는 다르지만 힘들 때 서로 위로해주고 실험실 생활을 하며 웃음을 줬던 경민이 오빠, 광철이 오빠, 준한이 오빠, 동원이, 유석이, 힘든 일도 많았지만 서로 위로했던 졸업 친구 안지, 졸업학기에 만나서 많은 시간을 함께하지는 못해서 아쉽지만 항상 반갑게 맞아주던 예은이, 대현이 모두들 감사했습니다. Thank you for being my lab friends, Nagarazan, Karthikeyan, and Antony. I hope that you guys are able to study well in Korea! Also, congratulation to my graduation friend, Jose!

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