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Rap1-dependent pathways coordinate cytokinesis in *Dictyostelium*

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ABSTRACT Cytokinesis is the final step of mitosis when a mother cell is separated into two daughter cells. Major cytoskeletal changes are essential for cytokinesis; it is, however, not well understood how the microtubules and actomyosin cytoskeleton are exactly regulated in time and space. In this paper, we show that during the early stages of cytokinesis, in rounded-up *Dictyostelium discoideum* cells, the small G-protein Rap1 is activated uniformly at the cell cortex. When cells begin to elongate, active Rap1 becomes restricted from the furrow region, where the myosin contractile ring is subsequently formed. In the final stages of cytokinesis, active Rap1 is only present at the cell poles. Mutant cells with decreased Rap1 activation at the poles showed strongly decreased growth rates. Hyperactivation of Rap1 results in severe growth delays and defective spindle formation in adherent cells and cell death in suspension. Furthermore, Rap mutants show aberrant regulation of the actomyosin cytoskeleton, resulting in extended furrow ingression times and asymmetrical cell division. We propose that Rap1 drives cytokinesis progression by coordinating the three major cytoskeletal components: microtubules, actin, and myosin II. Importantly, mutated forms of Rap also affect cytokinesis in other organisms, suggesting a conserved role for Rap in cell division.

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INTRODUCTION

Cell division is a fundamental process that is required for cell proliferation and differentiation of cell types. In anaphase, formation of the spindle apparatus pulls the chromosomes toward the poles of the dividing cell and triggers the beginning of cytokinesis, the final step in the separation of a mother cell into two daughter cells. Following the assembly of microtubule filaments in the expanding mitotic spindle, bundles of actin and non-muscle myosin filaments create a contractile ring that constricts the plasma membrane at the furrow region, while actin filaments are formed at the poles of the cells. This temporal and spatial regulation of the cytoskeleton is essential for the separation of the daughter cells (Glotzer, 2005; Kanada *et al.*, 2005; Wang, 2005). Most of the current models therefore incorporate these global, polar, and equatorial processes in

orchestrating the division process (Wang, 2005; Surcel *et al.*, 2010); it is, however, not well understood how they are coordinated in time and space. To investigate this in more detail, we used the model organism *Dictyostelium discoideum*.

Cell division is one example of cell polarization that depends on the organization of the cytoskeletal elements. Establishment and maintenance of eukaryotic cell polarization can be induced by small fluctuations of an asymmetric stimulus. A stimulus can be either intracellular, like signals from the dividing nucleus during cell division, or extracellular, such as chemical gradients during chemotaxis. Similar processes are involved in establishing polarity during chemotaxis and in regulating asymmetry during cytokinesis (Wang, 2005). Myosin II filaments and cortexillin are localized at the back of chemotactic cells and at the furrow of dividing cells, whereas PIP3 (phosphatidylinositol (3,4,5)-triphosphate), dynacortin, coronin, Scar, and active Ras and Rac are localized at the leading edge and poles during chemotaxis and cell division, respectively (Janetopoulos *et al.*, 2005; Sasaki *et al.*, 2007; King *et al.*, 2010; Surcel *et al.*, 2010). *Dictyostelium* Rap1 is a well-known component in establishing cell polarity and regulating cytoskeletal rearrangements during chemotaxis (Lee and Jeon, 2012). During chemotaxis, *Dictyostelium* Rap1 is involved in the regulation of adhesion, myosin II disassembly, and PI3K (phosphatidylinositol-3-kinase) activation (Kortholt and van Haastert, 2008; Lee and Jeon, 2012), all processes that are also critical for cytokinesis. Consistently,

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Abbreviations used: BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; DOX, doxycycline; GFP, green fluorescent protein; MTOCs, microtubule-organizing centers; RFP, red fluorescent protein.

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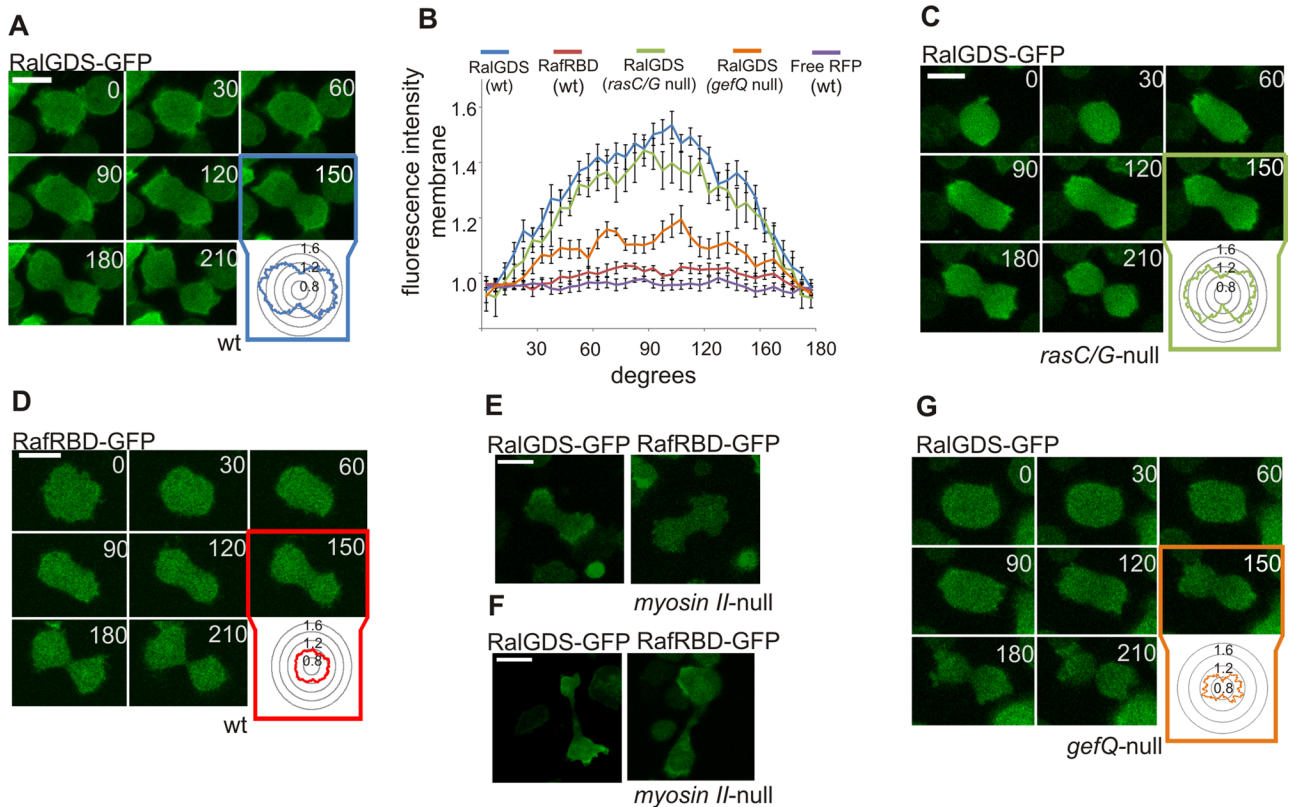


FIGURE 1: Dynamic Rap1 activation during cytokinesis. (A) Images of RalGDS-GFP (detecting active Rap) in dividing *Dictyostelium* wild-type cells. Inset: RalGDS-GFP fluorescence intensity was measured at the cell boundary around the circumference of the cell relative to the fluorescence intensity in the cytosol. (B) Quantification of the average fluorescence intensities of the indicated fluorescent markers along the cell membrane of dividing *Dictyostelium* cells presented as degrees from the cleavage furrow (see A, C, D, and G for representative images of the experiments). Error bars represent SEM. (C) Image of RalGDS-GFP in *rasC/rasG*-null cells. (D) Images of RafRBD-GFP (active Ras) in dividing *Dictyostelium* wild-type cells. Images of RalGDS-GFP or RafRBD-GFP in *myosin II*-null cells dividing by type B (E) and type C (F). (G) Image of RalGDS-GFP marker in *gefQ*-null cells. Scale bars: 10 μ m.

knockdown of *rap1* in *Dictyostelium* results in decreased growth rate and cell viability (Kang *et al.*, 2002). In this paper, we show that proper Rap1 activation is important for regulating cytokinesis progression in *Dictyostelium*. Rap1 is dynamically activated during cytokinesis; in the early stages of cytokinesis, Rap1 is activated uniformly at the cell cortex, where it regulates adhesion and contractile force, while at later stages Rap1 regulates adhesion and cytoskeleton dynamics at the cell poles. We propose a model in which Rap1 drives cytokinesis progression by coordinating global, polar, and equatorial changes of the three major cytoskeletal components: microtubules, actin, and myosin II.

RESULTS

Rap1 regulates several processes in moving cells, such as adhesion and cytoskeletal rearrangements, that are also important during cell division (Jeon *et al.*, 2007b; Kortholt and van Haastert 2008; Lee and Jeon, 2012). To investigate whether Rap1 plays a role during cytokinesis, we analyzed 1) Ras and Rap1 localization and activation in dividing *Dictyostelium* cells; 2) growth and cytokinesis of mutants with decreased or increased Rap1 activation; and 3) the role of Rap1 in coordinating microtubules, actin, and myosin II during cell division.

Dynamic Rap1 activation during *Dictyostelium* cytokinesis

Supplemental Figure S1A shows that N-terminal green fluorescent protein (GFP)-fused Rap1 is localized uniformly at the cell mem-

brane during both growth and cytokinesis of *Dictyostelium* cells. To monitor spatial activation of the protein, rather than its localization, we used the previously described molecular probe for active Rap1, RalGDS-GFP (Jeon *et al.*, 2007b; Kortholt and van Haastert, 2008). In unstimulated vegetative cells, Rap1 activation was observed at the cell cortex and in patches at the cell membrane that may correspond to macropinosomes (Supplemental Figure S1B). At the moment cells rounded up and entered cytokinesis, these RalGDS patches disappeared and Rap1 became uniformly activated at the cell cortex. Once the cell started to elongate, Rap1 activation was restricted to the sites that later became the poles of the dividing cell (Figure 1A). In dumbbell-shaped cells, the RalGDS marker was depleted from the furrow region, and strongly localized to the poles (Figure 1, A and B). RalGDS-GFP intensity at the membrane was measured around the circumference of cells (Figure 1B). The average fluorescence of RalGDS-GFP at the poles was 1.48 ± 0.12 ($n = 10$) times the cytosolic fluorescence, while the fluorescence at the furrow region (1.01 ± 0.12 , $n = 10$) was similar to that in the cytosol (Figure 1, A and B). This asymmetric Rap1 activation persisted until the moment the two daughter cells separated from each other.

Previous studies have shown that Rap activation at the leading edge of chemotactic *Dictyostelium* cells is completely dependent on heterotrimeric G-protein ($G\alpha 2\beta\gamma$) and RasG signaling (Bolourani *et al.*, 2008). In contrast, Rap1 activation during cytokinesis is not dependent on heterotrimeric G-protein signaling; dividing $G\beta$ -null

cells showed normal RalGDS localization at the poles (Supplemental Figure S1, C and D). Cells lacking *rasC* and *rasG* are unable to undergo chemotaxis and have severe growth defects in suspension culture (Tuxworth *et al.*, 1997); however, when these cells are grown on substrates, the timing and progression of cytokinesis is essentially the same as that of wild-type cells (Figure 1C). Importantly, *rasC/rasG*-null cells showed normal RalGDS-GFP accumulation at the poles of dividing cells (Figure 1B), with average levels at the poles and furrow of 1.40 ± 0.04 and 0.96 ± 0.13 , respectively (Figure 1, B and C; the numbers are not statistically different from wild-type cells).

To investigate whether Ras as well as Rap1 is activated during cytokinesis, we monitored Ras activation *in vivo* with the previously described GFP-RafRBD marker (Sasaki *et al.*, 2004; Kortholt and van Haastert, 2008). Vegetative wild-type cells expressing RafRBD showed Ras activation at dynamic membrane patches (Supplemental Figure S1E). When cells rounded up to enter cell division, Ras activation completely disappeared for 2–3 min (Figure 1D). Importantly, during this stage of cell division, we already observed very strong activation of Rap1 at the poles (Figure 1A). The calculated average levels of GFP fluorescence for the poles and furrow region were 1.01 ± 0.04 and 0.95 ± 0.04 times that of cytosolic fluorescence ($n = 10$; Figure 1, B and D). Only during late stages of cytokinesis, when the two daughter cells were almost separated, did RafRBD-GFP become slightly enriched at the poles, as has been described before (Sasaki *et al.*, 2007).

Rap and Ras activation during myosin-independent cytokinesis

Under normal conditions, wild-type *Dictyostelium* cells almost exclusively divide by a mechanism of cytokinesis called type A, which depends on the formation of a myosin contractile ring at the cell midzone (Fukui *et al.*, 1990). This type of cytokinesis is characterized by evenly sized daughter cells, and the time to complete the scission is ~3 min. Incidentally, wild-type cells grown on solid substrates divide without the formation of a myosin contractile ring (Uyeda *et al.*, 2000). Such myosin-independent cytokinesis is critically dependent on substrate attachment (Nagasaki *et al.*, 2002, 2009). Cells lacking *myosin II* are therefore only viable when grown on substrates. Analyses of RalGDS localization in *myosin II*-null cells revealed that Rap1 is strongly activated at the poles (Figure 1, E and F), indicating that spatial activation of Rap1 during cytokinesis is independent of myosin II.

There are two modes of this myosin-independent cytokinesis, called type B and type C (Neujahr *et al.*, 1997; Nagasaki *et al.*, 2009). Cytokinesis type B is spatiotemporally coupled to nuclear division, and the closing of the cleavage furrow is suggested to be passive and dependent on traction forces produced at the cell polar regions (Nagasaki *et al.*, 2002). During cytokinesis B, Rap1 was strongly activated at the poles, while no activation of Ras could be detected (Figure 1E). This shows that during both cytokinesis type A and B, which are coordinated with karyokinesis, Rap1 activation is independent of Ras. Interestingly, during cytokinesis type C, both Ras and Rap were activated at the poles (Figure 1F), resembling Rap and Ras activation at the leading edge during chemotaxis (Kortholt and van Haastert, 2008). This is consistent with the observation that cytokinesis type C is not coupled to nuclear division and is the result of different parts of one multinucleate cell crawling away from each other. This process thus resembles directional movement of cells (Uyeda *et al.*, 2000).

Together these data show that Rap1 but not Ras is activated at the poles of dividing *Dictyostelium* wild-type cells. In contrast to

chemotaxis, this spatial activation of Rap1 does not depend on Ras activity. Similar results are obtained for cells lacking *myosin II*, except for the unusual cytokinesis type C. In the next sections, we discuss the growth and cytokinesis of mutants with decreased or increased Rap1 activation.

Decreased Rap1 activation results in severe growth defects

Expression of antisense *rap1* mRNA in *Dictyostelium* cells results in decreased growth rate and cell viability (Kang *et al.*, 2002). Multiple attempts to disrupt *rap1* in *Dictyostelium* wild-type cells failed (Kang *et al.*, 2002). Therefore we tried to generate a conditional knockout by disrupting *rap1* in cells expressing Rap1 from an inducible extra-chromosomal plasmid or by knocking in the inducible promoter sequence in the genomic *rap1* promoter locus. Although a few clones were initially obtained, these cells were highly unstable and finally died.

GbpD and GefQ work together regulating Rap1 activation in vegetative cells (Plak *et al.*, unpublished data). Mutants cells lacking or overexpressing GbpD have normal cytokinesis and growth rates (Bosgraaf *et al.*, 2005). Cells overexpressing GefQ have severe cytokinetic defects in suspension and have limited ability to assemble myosin filaments (Mondal *et al.*, 2008). We determined the growth rate of *gefQ*-null cells and observed that mutant cells grow much more slowly than the parental strain, with doubling times in shaking suspension of 30 and 13 h, respectively. These *gefQ*-null cells show an almost 50% reduction of Rap1 activation at the poles of dividing cells (Figure 1, B and G). Together these results suggest that GefQ-mediated Rap activation is important for regulating cytoskeleton rearrangements during cell division.

Constitutive activation of Rap1 results in severe cytokinetic defects

Most of the current knowledge about Rap-mediated signaling in *Dictyostelium* comes from studies using constitutively active Rap1G12V mutants. Cells expressing Rap1G12V are large and flattened (Rebstein *et al.*, 1997), have strongly increased substrate adhesion, and exhibit chemotactic defects (Jeon *et al.*, 2007b). Because of these severe defects, we used here a doxycycline-inducible plasmid system (pDM310) to study the effect of Rap1G12V on cell growth and cell division (Veltman *et al.*, 2009). Seven hours after induction of Rap1G12V expression (Supplemental Figure S1F), cells showed the characteristic Rap1G12V phenotype and became larger and flattened, with an average cell area of $249 \pm 247 \mu\text{m}^2$ ($n = 180$), compared with $87 \pm 26 \mu\text{m}^2$ ($n = 180$) for uninduced control cells (Figure 2, A and B). The broad size distribution (Figure 2A) is caused by large variation in Rap1G12V expression levels within the cell population (Supplemental Figure S1G). This is the consequence of the variation in plasmid copy number per cell that is typically observed with the used inducible plasmid system (Veltman *et al.*, 2009; Veltman and van Haastert, 2013).

In addition to the large cell surface area, Rap1G12V cells have strongly increased substrate attachment. Because precise spatiotemporal regulation of cellular adhesion strength has recently been proposed to be important in successful closure of the cleavage furrow in mitotic HeLa cells (Bastos *et al.*, 2012), we investigated the closure of the cleavage furrow in Rap1G12V cells. Vegetative cells were observed during 30 h; wild-type cells did undergo approximately three division cycles, whereas 70% of the Rap1G12V cells did not manage to complete a single division cycle. The Rap1G12V cells that divided did so in a manner that was different from that of wild-type cells. As described in the next paragraph, two major types of cytokinetic defects are observed in the few Rap1G12V

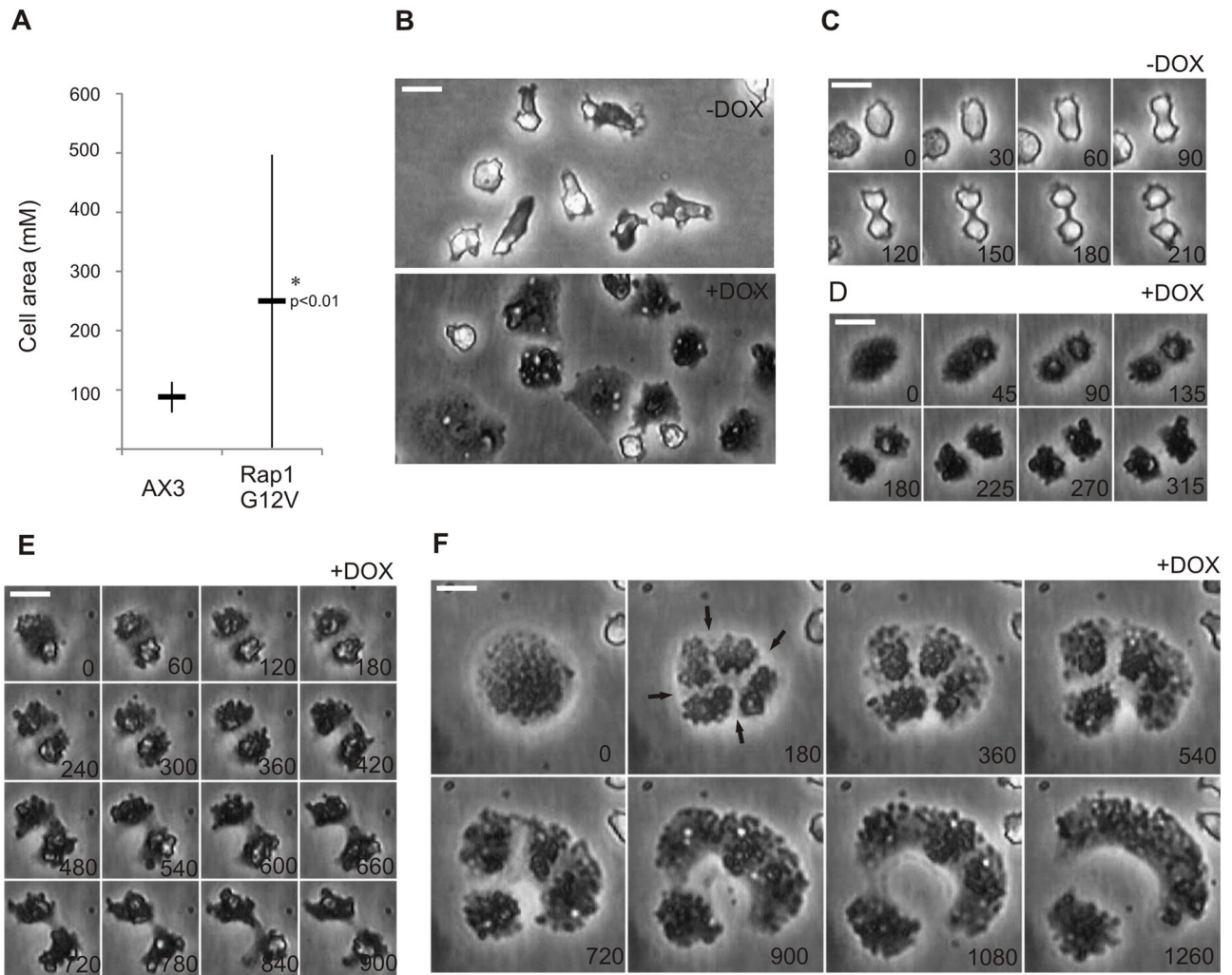


FIGURE 2: Hyperactivation of Rap1 leads to various cytokinetic defects on substrates. *Dictyostelium* Rap1G12V (+DOX) and control (–DOX) cells. Shown are (A) graph depicting the average cell size of vegetative grown cells and (B–F) images of cells undergoing cytokinesis on substrates; time in seconds is indicated. Arrows in F show the regions where the furrow is being formed. Scale bars: 10 μ m. * indicates significantly different from wild type with $p < 0.01$.

cells that completed cytokinesis: extended time for completing the scission and asymmetrical furrow ingression.

Wild-type cells dividing by myosin-dependent cytokinesis round up and slightly detach from the substrate at the moment they enter mitosis. The cells remain less attached throughout cytokinesis, as was observed by their bright appearance under phase-contrast microscopy (Nagasaki *et al.*, 2002; Figure 2C). A subset of Rap1G12V cells showed only a moderate adhesion and cell-spreading phenotype (cell area of $<250 \mu\text{m}^2$). These cells rounded up before entering cell division, but in contrast to wild-type cells, they did not detach but remained strongly attached to the substrate during cytokinesis. Often, two daughter cells of equal size were formed (Figure 2D), but complete closure of the furrow did take significantly longer for the Rap1G12V cells ($6.7 \pm 0.8 \text{ min}$, $n = 9$) compared with wild-type cells ($4.0 \pm 0.3 \text{ min}$, $n = 14$). In several other cells, we observed that the closing of the furrow appeared to be asymmetrical, with one side of the furrow ingressing much more efficiently than the other side of the furrow. The cells that divided in this way did not exhibit the canonical dumbbell shape (Figure 2E) and needed more than 15 min, four times more than wild type, to complete division.

Hardly any of the cells with high levels of Rap1G12V expression (cell area of $>250 \mu\text{m}^2$) were able to divide within the given time period. The few cells that entered the division process initially rounded up and started to create a furrow, but this process suddenly stopped, and the cells returned back to the vegetative state. Only after a delay of several hours did these cells round up again and initially form multiple furrows, but they finally finished cytokinesis and divided asymmetrically into two daughter cells (Figure 2F).

Rap1G12V cells are unable to grow in shaking cultures

Rap1G12V cells were incubated with growth medium in a shaken culture. During the first 24 h, cells were growing but at a reduced rate compared with control cells; the doubling time of induced Rap1G12V cells is 29 h, while uninduced Rap1G12V cells or wild-type cells grown in the presence of doxycycline (DOX) have a doubling time of 15 h. In contrast to Rap1G12V cells grown on solid substrate (discussed previously), the size of Rap1G12V cells grown in suspension for 24 h did not increase (Supplemental Figure S2A). The DNA content of the Rap1G12V cells at this time point is not different from the uninduced control or wild-type cells (Supplemental Figure S2B), suggesting that Rap1 hyperactivation does not

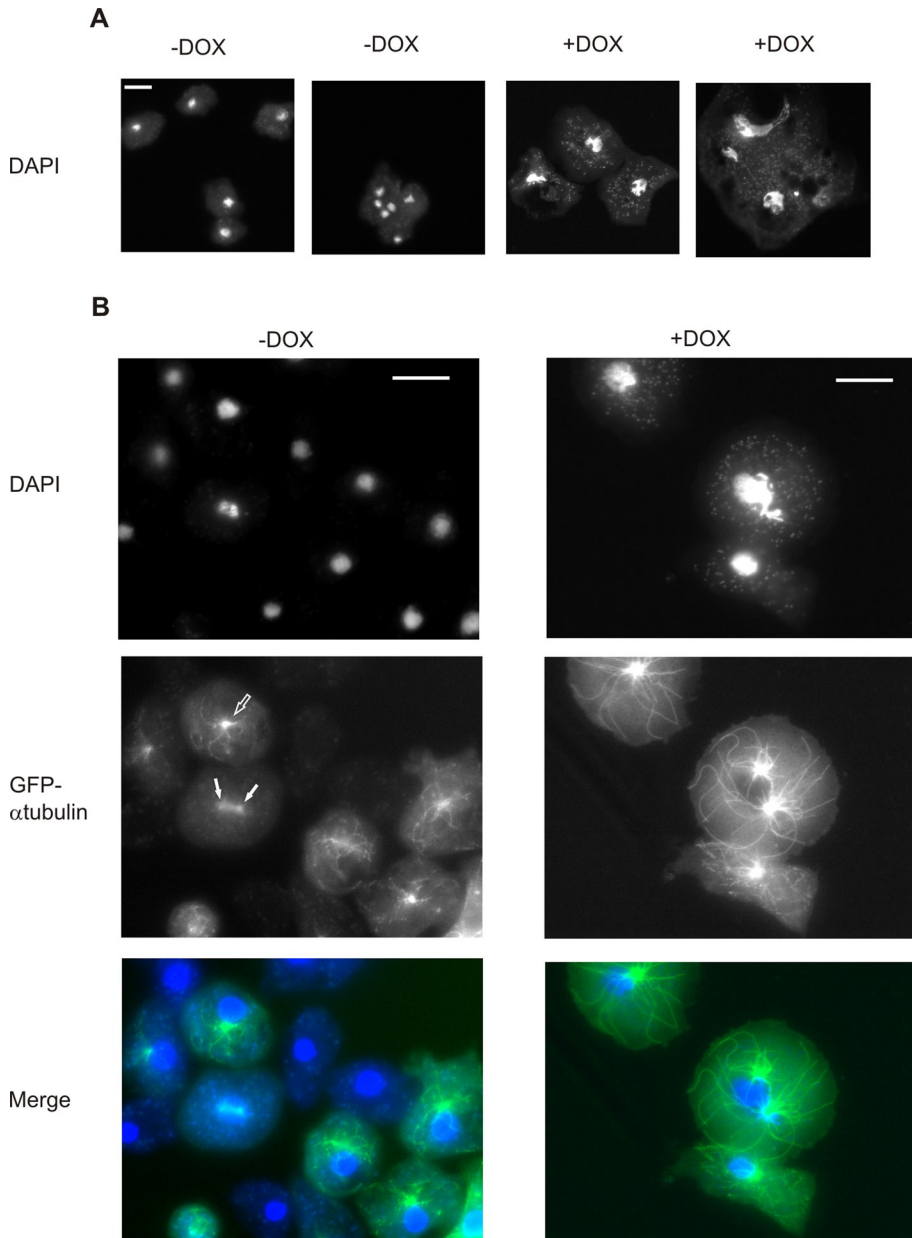


FIGURE 3: Size and shape of the nucleus and microtubular cytoskeleton are affected by expression of hyperactivated Rap1. Images of *Dictyostelium* Rap1G12V (+DOX) and control (–DOX) cells. Shown are (A) cells stained with DAPI and (B) cells coexpressing GFP- α -tubulin. Interphase MTOC is marked with open arrows, while mitotic structures are indicated with closed arrows. Scale bars: 10 μ m.

result in multinucleated cells. Nevertheless, after ~48 h in shaken suspension, the Rap1G12V-expressing cells started to lyse.

Overexpression of Rap1G12V does not lead to multinucleated cell phenotype but affects nuclear shape and size

Staining of Rap1G12V cells grown on substrates in the presence or absence of DOX for 48 h with 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye showed no significantly ($p > 0.01$) different average number of nuclei in Rap1G12V-expressing cells (1.34, $n = 114$, SEM = 0.08) compared with the uninduced control cells (1.16, $n = 90$, SEM = 0.06). Although hyperactivation of Rap does not influence the number of nuclei, it dramatically affects nuclear

size and shape. In control cells, the nuclei appeared small, round, and evenly sized both in single and in multinucleated cells (nuclear size of $8.6 \pm 2 \mu\text{m}^2$; Figure 3A). In contrast, Rap1G12V cells had irregularly shaped and much larger nuclei ($25.1 \pm 13.6 \mu\text{m}^2$). The small fraction of multinucleated Rap1G12V cells had nuclei of various shapes and sizes, and some of them were elongated or appeared to be connected (Figure 3A).

Together these results show that both reduced expression of Rap1 and hyperactivation of Rap1 lead to severe growth defects. Hyperactivation of Rap1 results in cytokinetic defects with misshapen nuclei. In the next sections, we describe the role of Rap1 in coordinating microtubules, actin, and myosin II during cell division.

Rap1 activation affects microtubule cytoskeleton organization

The above data show that Rap1G12V cells have severe growth and cytokinetic defects but are not multinucleated. This suggests that cells may fail to properly divide their nuclei at an early stage of cell division. Recently it was shown that an increased cellular area and decreased cell height can result in increased chromosome spreading after the breakdown of the nuclear envelope, which subsequently causes delays in chromosome capture and defects in spindle formation during the mitosis process (Lancaster *et al.*, 2013). Since *Dictyostelium* cells undergo closed mitosis, during which the nuclear envelope does not disassemble (Gräf *et al.*, 2000), we tested whether the increased size of the nucleus in Rap1G12V cells causes similar defects in spindle formation. Rap1G12V was coexpressed with GFP- α -tubulin, cells were stained with DAPI, and the distribution of nuclear DNA and the size of the microtubule cytoskeleton were analyzed. Wild-type interphase cells have microtubule-organizing centers (MTOCs) in close proximity to the nuclei, and in almost all cases (97%, $n = 102$), their number corresponds to the number of nuclei (Figure 3B, open arrow). Mitotic wild-type cells have clearly visible spindle microtubules, while nuclear DNA is condensed and starts to separate (Figure 3B, closed arrows). Rap1G12V cells have strong defects in microtubule cytoskeleton organization and abnormally shaped nuclei and MTOCs (Figure 3B). Furthermore, in 37% of the analyzed cells ($n = 96$), the number of MTOCs did not correspond to the number of nuclei present. Almost all of these Rap1G12V cells had a single, enlarged nuclei with two independent MTOCs attached to it, a phenotype that was never observed in wild-type cells. The two MTOCs are not connected by central-spindle microtubule filaments that are characteristic and essential for mitotic wild-type cells (Figure 3B).

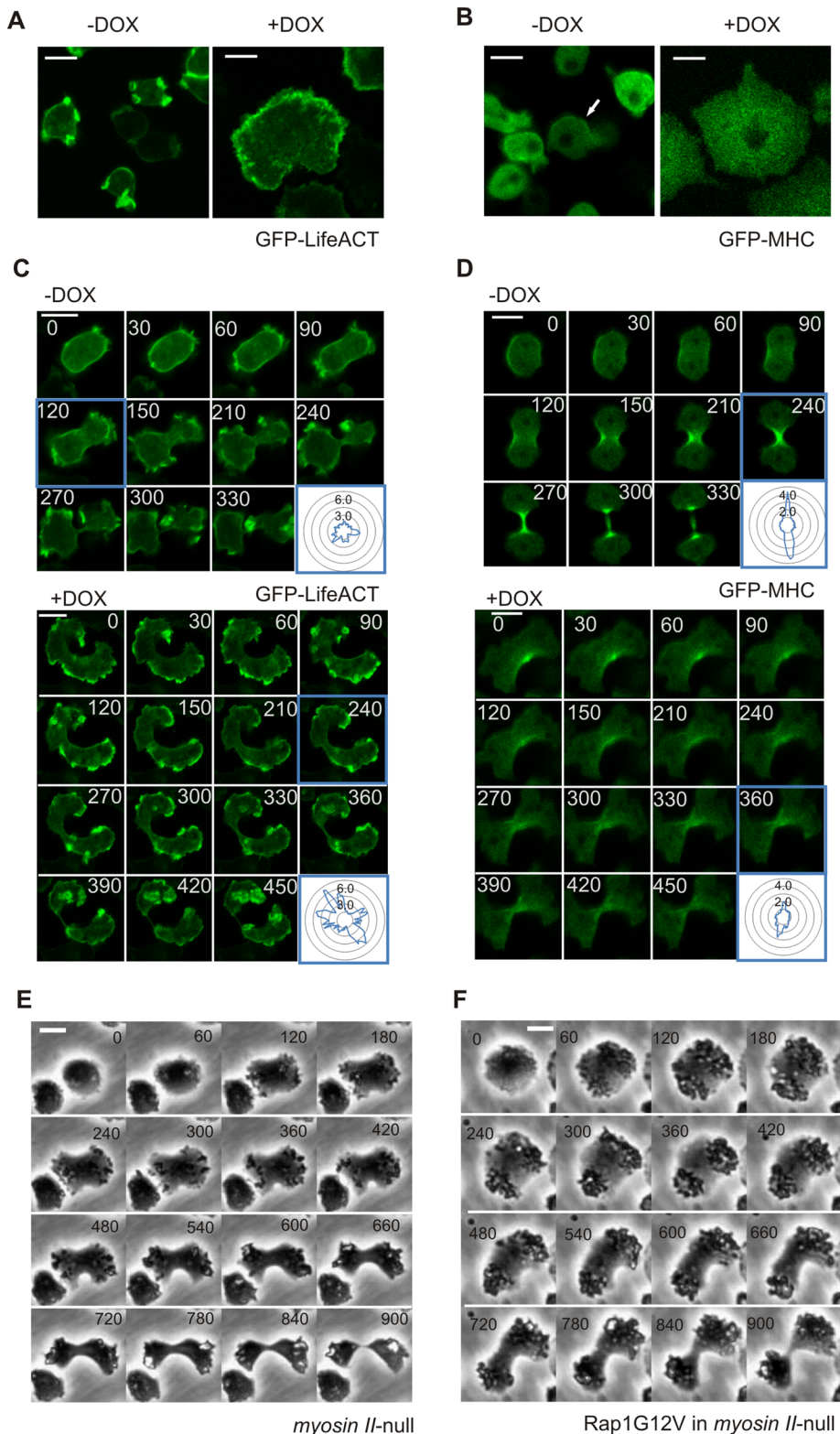


FIGURE 4: Rap1 regulates myosin and actin cytoskeleton dynamics. Images of vegetative induced (+DOX) or control (–DOX) cells expressing (A) Lifeact-GFP (detects filamentous actin) or (B) GFP-MHC. Localization of (C) Lifeact-GFP or (D) GFP-MHC during cytokinesis is shown. Inset: fluorescence intensity was measured at the cell boundary around the circumference of the cell relative to the fluorescence intensity in the cytosol. (E and F) Comparison of representative cell shape and timing of cells during cytokinesis. Shown are *myosin II*-null cells (E) and *myosin II*-null cells with Rap1G12V (F). Scale bars: 10 μ m.

Rap1 regulates myosin and actin rearrangements during cytokinesis

Dictyostelium Rap1 is an important regulator of both myosin and actin (Kortholt *et al.*, 2006; Kortholt and van Haastert, 2008; Jeon *et al.*, 2007b). Activation of Rap1 induces actin filaments when exposed to uniform chemoattractant stimulation (Mun and Jeon, 2012), while Rap1-mediated myosin heavy-chain phosphorylation results in myosin II filament disassembly at the leading edge of chemotactic cells (Jeon *et al.*, 2007b). Proper regulation of actin and myosin dynamics is essential for successful closure of the cytokinetic furrow (Faix *et al.*, 1996; O’Connell *et al.*, 2001; Gerisch *et al.*, 2004; Glotzer, 2005). Actin and myosin dynamics were monitored with the filamentous actin marker Lifeact (Riedl *et al.*, 2008) and GFP-MHC (Moore *et al.*, 1996), respectively. Vegetative control cells showed dynamic Lifeact patches at the cell boundary and weak cortical localization of myosin (Figure 4, A and B). After Rap1G12V expression was induced, the cortical myosin localization strongly diminished, and the cells showed a thick but dynamic actin-rich cortex around the whole cell boundary. During cell division, actin became enriched at the poles of both dividing wild-type and Rap1G12V cells, however, Rap1G12V cells showed larger amounts and more intense patches of F-actin at the cell boundary compared with the control cells (Figure 4C, bottom panels). Dividing control cells showed symmetrical localization of myosin at both sides of the cell furrow, which increased with time (Figure 4D, top panels). In contrast, dividing Rap1G12V cells showed an asymmetrical localization of myosin and even at late stages of cytokinesis the amount of myosin at the furrow was much lower than in control cells (Figure 4D, bottom panels). The side of the furrow with less myosin showed slower ingression, which consequently resulted in an asymmetrical cell shape during the division (Figure 4D, bottom panels). These data show that proper Rap1 activation is important for regulating the actomyosin cytoskeleton in response to nuclear division.

Both expression of Rap1G12V cells or disruption of myosin resulted in delayed closure of the cleavage furrow compared with wild-type cells (Figures 2, D–F, and 4E; Uyeda *et al.*, 2000). To further study the importance of Rap1-mediated cytoskeletal rearrangements during cytokinesis, we expressed Rap1G12V in *myosin II*-null cells. In the time frame during which 74% ($n = 86$) of the *myosin II*-null control cells completed

the cell cycle, only 26% ($n = 98$) of Rap1G12V *myosin II*-null cells divided. However, the timing and progression of the cytokinetic events were highly similar in Rap1G12V *myosin II*-null and *myosin II*-null cells (Figure 4, E and F). Interestingly, the defects of Rap1G12V *myosin II*-null cells were less severe than the defects of Rap1G12V cells, suggesting that the strong Rap1G12V phenotype partly depends on myosin II.

Rap1 affects cellular distribution of 14-3-3 in a RacE-dependent manner

We showed that Rap1G12V expression affects a number of cellular processes during mitosis progression, including defects in myosin assembly, adhesion, actin polymerization, and microtubule formation. Recently a RacE/14-3-3-dependent pathway was identified in *Dictyostelium* that links microtubules and cortical myosin to actin dynamics at the cortex (Zhou *et al.*, 2010). Although human 14-3-3 is an interaction partner for Rap proteins (Riou *et al.*, 2013), we were unable to detect any direct binding between activated *Dictyostelium* Rap1 and 14-3-3. However, because *Dictyostelium* Rap1G12V cells have increased Rac activation (Plak *et al.*, 2013), we hypothesized that Rap1 may cooperate with RacE and 14-3-3 in regulating *Dictyostelium* cytokinesis. Staining with 14-3-3-specific antibody, revealed weak cortical localization of 14-3-3 in nonmitotic wild-type cells (Figure 5A). Additionally, 14-3-3 and active Rap1 colocalized at the cell cortex (Figure 5B). A recent proteomic study suggested that *Dictyostelium* 14-3-3 is associated with centrosome during cell division (Reinders *et al.*, 2006), whereas microscopy data suggest that the protein is enriched at the polar cortex region of the dividing cell in late cytokinesis (Zhou *et al.*, 2010). Our data with both N-terminal or C-terminal GFP-fused 14-3-3 clearly show a dynamic cell cycle-dependent localization: in early mitotic stages, 14-3-3 is localized in the nucleus and subsequently at the centrosome (Figure 5C), where it colocalizes with Aurora kinase and, partially, α -tubulin (Figure 5D). Notably, we did not observe the previously described polar cortical localization of 14-3-3 (Zhou *et al.*, 2010). As the cytokinesis progressed the centrosomal localization disappeared, and at the late stages of cytokinesis, appeared to be lost completely (Figure 5, C and D). This centrosomal localization appears to be specific, as all dividing cells observed ($n = 22$) showed the same localization pattern. In Rap1G12V-expressing cells, 14-3-3 is mislocalized; vegetative Rap1G12V cells have increased amounts of 14-3-3 at the cortex compared with wild-type cells (Figure 5A). Most importantly, and in contrast to control cells, the cells that expressed low amounts of Rap1G12V and that were capable of dividing did not show nuclear or centrosomal localization of 14-3-3 during their division (Figure 5E).

It has previously been shown that cortex localization of 14-3-3 in vegetative *Dictyostelium* cells depends on the small G-protein RacE (Zhou *et al.*, 2010). We tested vegetative *racE*-null cells expressing Rap1G12V for their ability to translocate 14-3-3 to the cell cortex and detected much weaker cortical localization of 14-3-3 compared with control wild-type cells expressing Rap1G12V (Figure 5A). Interestingly, 14-3-3 was shown to be present in the nucleus during cytokinesis of Rap1G12V *racE*-null cells. Despite this nuclear localization of 14-3-3, *racE*-null/Rap1G12V cells still had the strong spreading phenotype and showed difficulties in completing cytokinesis (Figure 5, A and F). During cell division, the *racE*-null/Rap1G12V cells appeared to acquire polarity and elongated in the direction that was in accordance with the spindle axis, but the furrow was either ingressing slowly and asymmetrically or never formed (Figure 5F), which suggests defects at later stages of cytokinesis.

DISCUSSION

Coordinated changes in cellular adhesion, microtubule organization, and actin and myosin filament rearrangements contribute to cell division (Wang, 2005; Surcel *et al.*, 2010). We have shown here that the Rap1 activation pattern exhibits dynamical changes that allow for proper coordination of adhesion and cytoskeleton dynamics during the cell division process. Rap1 is specifically activated at the poles of dividing cells. Activation becomes detectable immediately after cells round up and persists while the mother cell elongates until the daughter cells are separated. The pathways that regulate activation of Rap1 during cytokinesis are different from those previously reported for chemotaxis. The heterotrimeric and monomeric G-protein signaling cascade that is indispensable for chemoattractant-induced Rap1 activation (Bolourani *et al.*, 2008), is not necessary for activation of Rap1 during cell division. GefQ, one of the three known GEFs for Rap1 in *Dictyostelium*, appears to be important for regulating Rap1 during cytokinesis. Furthermore, a recent study suggests that RapGAP9 is also crucial for cytokinesis progression (Mun *et al.*, 2014).

Knockdown of *rap1* severely affects the growth of *Dictyostelium* cells (Kang *et al.*, 2002), whereas expression of hyperactivated Rap1 results in multiple defects in progression of cytokinesis. Through comparison of the cytoskeleton in control and hyperactivated Rap1 cells, a picture is emerging on the complex role of Rap during cytokinesis.

At the mitotic entry, wild-type cells round up and detach from the substrate, which is associated with moderate and uniform Rap1 activation at the cell boundary. At this stage, the mitotic spindle assembles and designates the division plane, which allows for entry to cytokinesis. Previously it was shown that, in *Dictyostelium*, 14-3-3 links microtubules and cortical myosin to actin dynamics (Zhou *et al.*, 2010). We showed here that, in early mitotic control cells, 14-3-3 is localized in the nucleus and subsequently at the centrosome. Hyperactivation of Rap1 leads to accumulation of 14-3-3 at the cell cortex and prevents it from localizing to the mitotic spindle during the interphase. Our data suggest that this aberrant 14-3-3 localization is mediated by the small G-protein RacE. We have not been able to demonstrate a direct interaction between 14-3-3 and Rap1; it is thus unlikely that Rap1 directly regulates 14-3-3 localization. It has been shown that 14-3-3 is localized to the cortex by binding to several actin cross-linking proteins (for a review, see Sluchanko and Gusev, 2010). RacE regulates cortical localization of several global actin cross-linkers and is important for 14-3-3 localization during cytokinesis (Zhou *et al.*, 2010). Finally, we have shown that vegetative *racE*-null cells expressing Rap1G12V did not show the aberrant cortical localization of 14-3-3 when compared with wild-type cells expressing Rap1G12V, suggesting that RacE functions downstream of Rap1 and upstream of 14-3-3.

At the beginning of cytokinesis, activation of Rap1 is likely spatially regulated by cell cycle-activated GEFs (guanine exchange factors) and GAPs (GTPase-activating protein). Cells lacking *gefQ* showed reduced Rap1 activation at the poles of the dividing cells and reduced growth, while hyperactivation of GefQ resulted in decreased myosin assembly and defects in cytokinesis (Mondal *et al.*, 2008). Consistently, the few Rap1G12V cells that did enter the cell cycle failed to properly regulate the actomyosin cytoskeleton, resulting in extended furrow ingression times and asymmetrical cell division. The timing and progression of cytokinesis in Rap1G12V *myosin II*-null cells is highly similar to that of *myosin II*-null cells, however, far fewer Rap1G12V/*myosin II*-null entered the cell cycle. Furthermore, expression of Rap1G12V in wild-type cells reduced the growth rate in shaking suspension but, in contrast with *myosin*

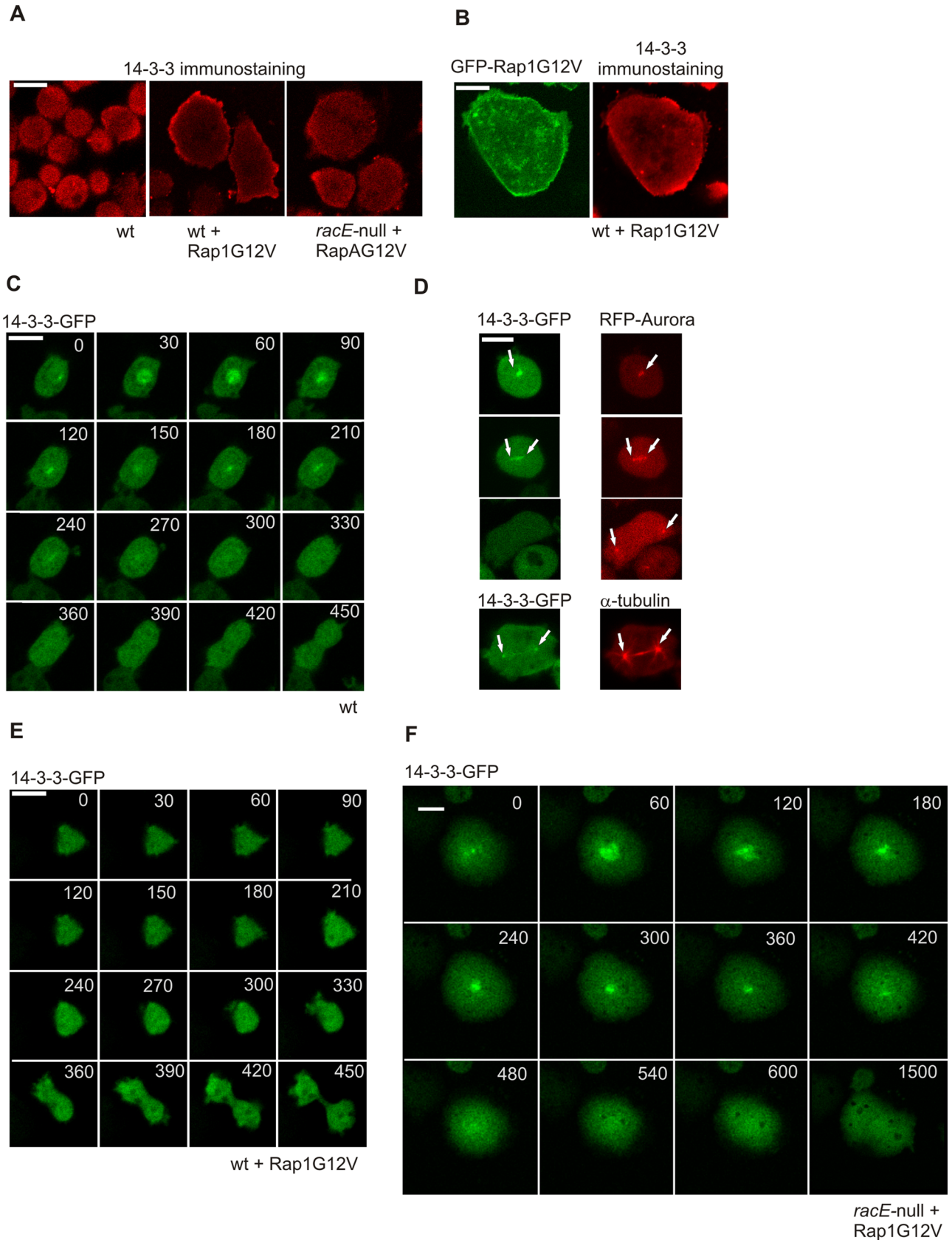


FIGURE 5: Rap1G12V affects 14-3-3 localization in RacE-dependent manner. (A) Images of immunostained 14-3-3 protein in the indicated cell strains. (B) Images showing colocalization of GFP-Rap1G12V and 14-3-3 in fixed *Dictyostelium* cells. (C) 14-3-3-GFP localization in dividing wild-type cells. (D) Colocalization of 14-3-3 with Aurora-mRFP or α -tubulin. 14-3-3 localization in (E) dividing Rap1G12V and (F) *racE*-null/Rap1G12V cells. Time is indicated in seconds. Scale bars: 10 μ m for all panels.

//-null cells, did not result in multinucleated cells. Together these data suggest that Rap-mediated actin/myosin rearrangements are not involved in regulating the entry to mitosis but are important for proper progression of the process to separate the emerging daughter cells. We therefore propose that Rap1 activation at the poles promotes adhesion and cell polarization by inducing F-actin remodeling through pathways that most likely include PI3K and Rac proteins and by inhibiting myosin assembly at the poles through its effector Phg2 (Jeon *et al.* 2007a; Kortholt and van Haastert, 2008; Lee and Jeon, 2012). At the same time, low levels of Rap1 activation in the equatorial zone of the dividing cell cause decreased adhesion and allow for myosin filament assembly. Those two processes promote contractile ring formation and closing of the cytokinetic furrow. Consistent with this model, Lancaster *et al.* (2013) recently showed that the cortical actin cytoskeleton does not contribute to spindle formation in mitotic human cells but is crucial for progression of cytokinesis and for maintaining proper cortical tension in cells grown in physically constrained tissues.

Previously it was shown that levels of activated Rap are tightly controlled during the progression of cell division in HeLa cells (Dao *et al.*, 2009). Furthermore, Rap hyperactivation in both *Drosophila* embryonic cells and human cell lines causes, as in *Dictyostelium*, defective spindle positioning (Carmena *et al.*, 2011; Lancaster and Baum, 2014). This strongly suggests a conserved and important role for Rap in regulating cell cycle progression and cytokinesis.

MATERIALS AND METHODS

Strains and constructs

AX3 *Dictyostelium* cells were used as the wild-type strain. Cells were grown in HL5-C media (Formedium, Norfolk, UK) either on Nunclon-coated Petri dishes or in shaking flasks. For selection, the medium was supplemented with the appropriate antibiotics; 10 µg/ml G418, 50 µg/ml hygromycin B, 10 µg/ml DOX, or 10 µg/ml blasticidin S. Knockout strains of *gβ* (DBS0236531), *rasC/rasG* (DBS0236858), *myosin* (DBS0236387), and *racE* (DBS0235413) genes were provided by the *Dictyostelium* stock center (Fey *et al.*, 2013). Rap1G12V was amplified by PCR, using the previously described pVE11 Rap1G12V plasmid as a template (Rebstein *et al.*, 1997). The obtained PCR fragments were digested with *Bam*HI and ligated into the *Bgl*II site of the previously characterized *Dictyostelium* extrachromosomal Tet-ON plasmids pDM310 or pDM334 (N-terminal GFP fusion; Veltman *et al.*, 2009). The *14-3-3*, *aurora*, and *α-tubulin* genes were amplified from *Dictyostelium* cDNA by PCR, digested with *Bam*HI, and cloned into the *Bgl*II site of a pDM317 (N-terminal GFP fusion), pDM318 (N-terminal red fluorescent protein [RFP]), or pDM323 (C-terminal GFP fusion) (Veltman *et al.*, 2009). A *rap1* knockout construct was created by amplifying the genomic sequence of Rap1 and ligating it to pBlue vector. Subsequently a blasticidin resistance cassette (*bsr*) was inserted within the *rap1* gene, and the final construct was amplified by means of PCR. *Dictyostelium* cells were transformed by means of electroporation.

Immunocytochemistry and DAPI staining

Cells were allowed to grow on glass-bottom dishes (MatTek) and were washed with PB buffer (10 mM KH₂PO₄/Na₂HPO₄, pH 6.5) before fixation. Cells were fixed by incubation for 15 min with PB containing 4% paraformaldehyde, washed with PB, and permeabilized by two 10-min incubations in PB containing 0.1% Triton X-100. Subsequently cells were blocked for 30 min in PB containing 1% wt/vol bovine serum albumin (BSA) and 0.1% Tween, incubated with 14-3-3 antibody (ab77187 [Abcam, Cambridge, UK], 1:200 diluted in 1% BSA, 0.1% Tween PB) or *α-tubulin* antibody (sc53029 [Santa Cruz,

Heidelberg, Germany], 1:50 diluted in 1%BSA, 0.1% Tween PB) for 1 h, washed three times with PB buffer, and finally incubated for 1 h in the dark with a secondary antibody (ab150130 [Abcam] or sc2782 [SantaCruz], 1:200 dilution with 1% BSA, 0.1% Tween, PB). Staining of DNA was performed with 20 µg/ml DAPI solution in PB for 10 min.

Microscopy

A Motic AE31 (Motic, Wetzlar, Germany) bright-field inverted microscope with an LWD PH20x/0.4 NA objective was used to monitor growth of *Dictyostelium* on Nunclon-coated plates. The images were recorded every 8 s with a digital camera (ScopeTek DCM130) using VirtualDub software (www.virtualdub.org) and Indeo video 5.10 compression (Ligos). The field of observation was 550 × 440 µm.

Fluorescence in fixed or living *Dictyostelium* cells was monitored using a confocal laser-scanning microscope (LSM 510 META-NLO; Carl Zeiss Microimaging) equipped with a 63x/NA 1.4 objective (Plan-Apochromatic; Carl Zeiss Microimaging). For excitation of the fluorochromes GFP (S65T variant) and Alexa Fluor 555, a 488-nm argon/krypton laser and a 543-nm helium laser were used, respectively. The fluorescence was filtered through a BP500-530 IR and a LP560 filter and was detected by a photomultiplier tube. The field of observation was 202 × 202 µm, and for in vivo observation of dividing *Dictyostelium* cells, the images were recorded with a scan time of 15 s.

DAPI-stained cells were imaged with a wide-field fluorescence Zeiss Axio Observer Z1 microscope (Zeiss, Jena, Germany) equipped with an EC-Plan-Neofluar 100x/1.3 NA objective and CoolSNAP HQ2 Camera (Roper Scientific, Martinsried, Germany). A 365- to 395-nm band-pass excitation filter, a 420-nm dichroic mirror, and a 435- to 485-nm band-pass emission filter were used. The field of observation was 271 × 202 µm. All images were analyzed using ImageJ software (Abramoff *et al.*, 2004).

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