Published in final edited form as:

Oncogene. 2010 November 11; 29(45): 6040–6050. doi:10.1038/onc.2010.328.

RhoA and RhoC Are Both Required for the ROCK II-Dependent Promotion of Centrosome Duplication

Masayuki Kanai[1], **Matthew S. Crowe**[1], **Yi Zheng**[2], **George F. Vande Woude**[3], and **Kenji Fukasawa**[1,*]

[1]Molecular Oncology Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL 33612, USA

[2]Division of Experimental Hematology and Molecular, Developmental Biology Program, Children's Hospital Research Foundation, University of Cincinnati, Cincinnati, OH 45229, USA

[3]Van Andel Research Institute, Grand Rapids, MI, USA

Summary

CDK2-cyclin E triggers centrosome duplication, and nucleophosmin (NPM/B23) is found to be one of its targets. NPM/B23 phosphorylated by CDK2-cyclin E acquires a high binding affinity to Rho-associated kinase (ROCK II), and physically associates with ROCK II. The NPM/B23-binding results in super-activation of ROCK II, which is a critical event for initiation of centrosome duplication. The activation of ROCK II also requires the binding of Rho small GTPase to the auto-inhibitory region; hence the availability of the active Rho protein is an important aspect of the centrosomally localized ROCK II to properly initiate centrosome duplication. There are three isoforms of Rho (RhoA, B, and C), all of which are capable of binding to and priming the activation of ROCK II. Here, we investigated which Rho isoform(s) are involved in the activation of ROCK II in respect to the initiation of centrosome duplication. We found that both RhoA and RhoC, but not RhoB, were required for initiation of centrosome duplication, and over-activation of RhoA as well as RhoC, but not RhoB, promoted centrosome duplication and centrosome amplification.

Keywords

Centrosome; Rho; ROCK II; CDK2; Cyclin E; nucleophosmin; NPM/B23; Met

Introduction

The centrosome is a small non-membranous organelle composed of a pair of centrioles and surrounding amorphous pericentriolar materials. The function of the centrosome is to nucleate and anchor microtubules, and thus plays a key role in organizing microtubule networks during interphase and spindle formation during mitosis (reviewed in Doxsey,

*To whom correspondence should be addressed: Telephone: 1-813-745-2824, FAX: 1-813-979-7265, kenji.fukasawa@moffitt.org.

2001; Fukasawa, 2007). The centrosome, like DNA, duplicates once during the cell cycle, and duplication of these two organelles proceeds in coordination: centrosome duplication starts at the G1/S boundary by physical splitting of the paired centrioles, followed by formation of procentrioles in the vicinity of each preexisting centriole and progressive recruitment of centrosomal proteins, resulting in generation of two mature centrosomes by the late G2 phase of the cell cycle (reviewed in Hinchcliffe and Sluder, 2002). In mitosis, these two duplicated centrosomes direct the formation of bipolar mitotic spindles, and the bipolarity of the mitotic spindles is critical for accurate chromosome transmission into daughter cells during cytokinesis. The centrosome duplication process is highly regulated to ensure one and only one duplication of the centrosome in a single cell cycle, and thus abrogation of the regulatory mechanism leads to multiple rounds of duplication in a single cell cycle, resulting in the generation of more than two centrosomes (centrosome amplification). The presence of amplified centrosomes disrupts the proper formation of the bipolar mitotic spindles, leading to unbalanced chromosome segregation (reviewed in Fukasawa, 2007). Chromosome instability accelerates tumor progression by introducing multiple genetic alterations responsible for acquisition of malignant phenotypes, and centrosome amplification is thought to be the major cause of chromosome instability in cancer cells (reviewed in D′Assoro *et al.*, 2002; Fukasawa, 2005).

Coupling of the initiation of centrosome duplication and DNA replication is achieved by the late G1-specific activation of cyclin-dependent kinase 2 (CDK2)-cyclin E. CDK2-cyclin E, a known initiator of DNA replication (reviewed in Morgan, 1997; Reed, 1997), also triggers the initiation of centrosome duplication (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999; Matsumoto *et al.*, 1999; Tarapore *et al.*, 2002). CDK2-cyclin E appears to target multiple proteins to trigger initiation of centrosome duplication, including nucleophosmin (NPM/B23), Mps1 kinase, and CP110 (Okuda *et al.*, 2000; Fisk and Winey, 2001; Chen *et al.*, 2002). NPM/B23, a molecular chaperoning protein, phosphorylated by CDK2-cyclin E (Tokuyama *et al.*, 2001) binds to and super-activates ROCK II kinase, and the super-activation of ROCK II by NPM/B23 is one of the critical events for initiation of centrosome duplication (Ma *et al.*, 2006). Activation of ROCK II also requires the binding of Rho small GTPase. ROCK II has an autoinhibitory C-terminal region, which folds back to physically interact with the N-terminal kinase region, leading to inhibition of the kinase activity, and binding of GTP-bound Rho (Rho-GTP) to the C-terminal Rho-binding domain releases the kinase domain from this negative regulatory mechanism (Matsui *et al.*, 1996; Nakagawa *et al.*, 1996). There are three Rho isoforms: RhoA, RhoB and RhoC. Despite of the high homology among them, they appear to participate in different cellular events/functions due to the differences in their expression patterns, their activation patterns by the upstream regulatory proteins, their binding activities/affinities to their target proteins and their sub-cellular localization patterns (reviewed in Wheeler and Ridley, 2004). In cancer, RhoA and RhoC are frequently overexpressed/over-activated, while RhoB is often down-regulated. It has been reported that RhoA and RhoC contribute proliferation and metastasis of cancer cells, while RhoB inhibits invasion and metastasis (reviewed in Vega and Ridley, 2008).

Because all Rho isoforms are able to bind to and activate ROCK II, we investigated which Rho isoform(s) are responsible for activation of ROCK II to promote centrosome duplication, and found that both RhoA and RhoC, but not RhoB, play critical roles.

Materials and Methods

Cell culture and transfection

NIH3T3 cells and primary mouse skin fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in an atmosphere containing 10% CO_2 at 37°C. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Plasmids and antibodies

The RhoA, RhoB, and RhoC mutants were generated by the PCR-based mutagenesis. The siRNA sequences were chosen based on the previously published studies, in which when the sequences were of human cDNA, the corresponding mouse cDNA sequences were used. The sequences of RhoA siRNA #1 (5′-GCAGGTAGAGTTGGCTTTA-3′) and RhoC siRNA #1 (5′-GACTACGATCGCCTGCGGC-3′) were adopted from the human siRNA sequences used in the study by Simpson *et al.* (2004). The sequence of RhoA siRNA #2 (5′-AAGGCAGAGATATGGCAAA -3′) was from the study by Wang *et al.* (2003), and the sequence of RhoC siRNA #2 (5′-GGAGAGAGCTGGCCAAGAT-3′) was from the human sequence published by Kabuyama *et al.* (2009). The RhoB siRNA sequence (5′-CCGGTTCGAGAACTATGT-3′) was taken from the human RhoB siRNA sequence used by Canguilhem *et al.* (2005). The ROCK II siRNA sequence was described previously (Ma *et al.*, 2006). The siRNA sequences were inserted into the pSuper vector (Oligoengine, Seattle, WA, USA).

The antibodies used in this study are: anti-RhoA, anti-RhoB, anti-RhoC, anti-γ-tubulin, anti-centrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GFP, anti-BrdU (Invitrogen, Carlsbad, CA, USA), anti-ROCK II (BD Biosciences, San Jose, CA, USA), and anti-β-actin (Sigma, St. Louis, MO, USA) antibodies.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Ma *et al.*, 2006). Briefly, cells were lysed in SDS/NP-40 lysis buffer. The lysates were heat-denatured, resolved by SDS-PAGE, and subjected to immunoblotting. The antibody-antigen complex was visualized by ECL chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

Indirect Immunofluorescence

Cells were fixed with 10% formalin/10% methanol for 20 min (or 100% cold methanol for 20 min at -20°C for centrin staining), and were permiabilized in 1% NP-40 in PBS. Cells were then blocked by 10% normal goat serum in PBS, and incubated with primary followed by secondary antibodies. The DNA was counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). For ROCK II immunostaining, because it is difficult to distinguish the ROCK II signals at centrosomes due to the ubiquitous presence of ROCK II (Ma *et al.*, 2006), the

transfected cells were briefly extracted prior to fixation with 0.1 % TritonX-100 in PBS. The cells were then fixed and subjected to immunostaining. Cells were examined under a fluorescence microscope (Zeiss Automated Upright Fluorescent Microscope, or Zeiss Automated Inverted Fluorescent Microscope).

Results

RhoA and RhoC, but not RhoB, are involved in the regulation of centrosome duplication

The activation of ROCK II requires the binding of activated Rho (Rho-GTP) to the C-terminal autoinhibitory region. Because all Rho isoforms (RhoA, B, and C) are capable of binding to and activate ROCK II, we tested which Rho isoform(s) are responsible for the ROCK II-associated promotion of centrosome duplication. To this end, we examined how depletion of each Rho isoform affects on the efficiency of centrosome duplication by the centrosome re-duplication assay. When centrosome duplication-permissive cells (*i.e.*, cells with functionally defective p53 or p53-dependent checkpoint pathway) are arrested by exposure to DNA synthesis inhibitors such as aphidicolin (Aph), centrosomes continue to re-duplicate without DNA synthesis, resulting in generation of more than two centrosomes (centrosome amplification) (Balczon *et al.*, 1995; Tarapore *et al.*, 2001). NIH3T3 cells carried in our laboratory are partially defective in the p53-dependent checkpoint pathway, and thus suitable for this assay. NIH3T3 cells were transfected with the siRNA sequence specific for each Rho isoform together with a plasmid containing green fluorescent protein (GFP) and neomycin resistant genes as selection markers. After a drug-selection, >90% of the surviving cells showed successful transfection (identified by the positive GFP) (data not shown). The surviving cells were examined for the expression of the respective Rho isoforms by immunoblot analysis (Fig. 1A). All isoforms were successfully silenced to <10% of the normal levels, and the siRNA sequence for each Rho isoform was highly specific: no significant reduction of the levels of other isoforms was detected (also see Supplemental Information, Fig 1). However, it should be noted here that, as reported previously (Ho *et al.*, 2008), we also observed an increase in the level of RhoB in cells silenced for RhoA.

These siRNA-transfected cells were subjected to the centrosome re-duplication assay. Depletion of both RhoA and RhoC resulted in suppression of centrosome re-duplication, while depletion of RhoB had no effect (Fig. 1B, representative immunostaining images of cells depleted for RhoA and RhoC are shown in Fig. 1C), implicating both RhoA and RhoC, but not RhoB, in the regulation of centrosome duplication. However, when the constitutively active mutant RhoA (Glu63 → Leu63; RhoA-L63) was introduced into cells silenced for RhoC expression, centrosome duplication was no longer suppressed (Fig. 1D). Similarly, centrosome duplication was no longer suppressed in the cells silenced for RhoA expression when RhoC-L63 was introduced (Fig. 1D). Thus, the function of RhoA may be readily compensated by RhoC overexpression/over-activation, and *vice versa*. Alternatively, RhoA and RhoC may act on the same target protein, and introduced RhoA or RhoC may compensate the shortage of the intracellular level of Rho required for initiation of centrosome duplication in cells depleted for RhoC or RhoA, respectively. In contrast to RhoA and RhoC, overexpression of RhoB-L63 failed to restore the centrosome re-

duplication potential in the cells silenced for RhoA as well as RhoC (Supplemental Information: Fig 2), further indicating that RhoB is not involved in the regulation of centrosome duplication during the Aph-induced arrest.

We next tested whether RhoA and RhoC are also involved in the regulation of centrosome duplication during the normal cell cycle. For this experiment, we used primary mouse skin fibroblasts (MSFs), which show the near perfect coupling of initiation of centrosome duplication and DNA replication. MSFs were first serum-starved, and transfected with RhoA as well as RhoC siRNA sequences. Cells were then serum-stimulated, and the rates of centrosome duplication and S-phase entry (BrdU-incorporation) were monitored for 35 hrs (Fig. 1E). There was no noticeable change in the rate of S-phase entry in the RhoA-siRNA and RhoC-siRNA cells. However, the initiation of centrosome duplication was dramatically delayed in the RhoA-siRNA and RhoC-siRNA cells compared with the control cells, indicating that both RhoA and RhoC are required for initiation for centrosome duplication during the normal cell cycle. We also tested whether depletion of RhoB affects the initiation of centrosome duplication during the normal cell cycle. Expectedly, the rates of centrosome duplication and S-phase entry of the cells silenced for RhoB expression were found to be similar to those of the control cells (data not shown).

To corroborate the above findings, we tested how functional inhibition of each Rho isoform affects centrosome duplication. To this end, we transfected the GFP-tagged dominant negative Rho mutant (Thr19 → Agn; N19) of each isoform into NIH3T3 cells. After confirming the comparable expression levels of the N19 mutants (Fig. 2A, lanes 2, 4, 6), the transfected cells were subjected to the centrosome re-duplication assay. Similar to the siRNA-mediated silencing study, expression of RhoA-N19 and RhoC-N19 resulted in suppression of centrosome duplication, while expression of RhoB-N19 had no effect (Fig. 2B; representative immunostaining images of RhoA-N19-, RhoB-N19- and RhoC-N19-transfected cells are shown in Fig. 2C, panels a-c). These results further demonstrate that both RhoA and RhoC, but not RhoB, are required for initiation of centrosome duplication.

Overexpression/overactivation of RhoA and RhoC, but not RhoB, promotes centrosome duplication

We next tested how exogenously introduced constitutively active mutant Rho proteins affect on centrosome duplication. Rho protein whose Gln63 is replaced to Leu (L63) is resistant to GTP-GDP exchange, and thus remains as a GTP-bound active form (Wang *et al.*, 2003). NIH3T3 cells were transfected with GFP-tagged L63 mutant of each isoform. All L63 mutants are expressed at comparable levels (Fig. 2A, lanes 3, 5, 7). The transfected cells were subjected to the centrosome re-duplication assay. Expression of RhoA-L63 and RhoC-L63 both resulted in an increase in the frequency of centrosome re-duplication compared with the control cells, while such an increase was minimal in cells transfected with RhoB-L63 (Fig. 2B; representative immunostaining images of RhoA-L63-, RhoB-L63- and RhoC-L63-transfected cells are shown in Fig. 2C, panels d-f). To examine the structural integrity of the amplified centrosomes, the transfected cells were also co-immunostained for γ-tubulin and centrin (Fig. 2D). Centrin closely associates with centrioles, and thus immunostaining of centrin allows visualization of the paired centrioles within the centrosome (review in

Salisbury, 2007). Each amplified centrosome contains a pair of centrioles, indicating that amplified centrosomes are not due to fragmentation of centrosomes. These results further indicate that, although RhoB may have a minimal activity to promote centrosome duplication, RhoA and RhoC are actively involved in the regulation of the initiation of centrosome duplication. Moreover, the inability of RhoB to promote centrosome duplication is not due to the failure or inefficiency of being activated.

RhoA and RhoC, but not RhoB, localize to centrosomes

We have previously shown that ROCK II localizes to centrosomes, and controls centrosome duplication at the centrosome (Ma *et al.*, 2006). Although all GTP-bound Rho isoforms are known to bind to and activate ROCK II, the abilities of RhoA and RhoC, but not RhoB, to promote centrosome duplication may be attributed to their abilities to localize to centrosomes. To test this possibility, NIH3T3 cells were transfected with GFP-tagged constitutively active (L63) and dominant negative (N19) mutants of RhoA, RhoB and RhoC, and co-immunostained with anti-γ-tubulin and anti-GFP antibodies. The localization of RhoA-L63 and RhoC-L63 were readily observed at centrosomes as well as at pericentrosomal regions, while RhoB-L63 was not detected at or around centrosomes (Fig. 3A, b-d), suggesting that the inability of RhoB to promote centrosome duplication may be due to a failure to localize to centrosomes. In contrast to GTP-bound Rho, all the dominant negative mutants (RhoA-N19, RhoB-N19 and RhoC-N19) were negligible at centrosomes (Fig. 3A, e-g). Thus, GTP-bound forms of RhoA and RhoC, but not GDP-bound forms, appear to efficiently localize to centrosomes.

We also tested whether centrosomal localization of Rho depends on the presence of ROCK II by analyzing the RhoA-L63 mutant expressed in the cells silenced for ROCK II. We found that GFP-RhoA-L63 was still able to localize to centrosomes (data not shown), indicating that centrosomal localization of Rho does not depend on the presence of ROCK II. This is not unexpected, considering that other Rho-binding proteins (*i.e.*, ROCK I) are known to localize to centrosomes (Chevrier *et al.*, 2002).

Because GTP-bound forms of RhoA and RhoC localize to the centrosome, we next tested whether centrosomal localization of ROCK II depends on RhoA or RhoC. NIH3T3 cells were transfected with RhoA siRNA, RhoC siRNA, or FLAG-tagged L63 constitutively active mutant, L40 ROCK II activation defective mutant and L40/L63 double-mutant of RhoA and RhoC. For all the transfections, GFP-centrin was co-transfected as a transfection as well as a centriole marker. RhoA-N19 dominant negative mutant was used as a control. The transfected cells were briefly extracted prior to fixation, and subjected to immunostaining with anti-GFP and anti-ROCK II antibodies. We found no recognizable difference in centrosomal localization patterns of ROCK II among cells silenced for RhoA or RhoC and those cells expressing various Rho mutants (for representative immunostaining images, see Fig. 3B), indicating that centrosomal localization of ROCK II is independent of Rho. This result agrees with our previous findings that the N-terminal region (a.a. 421-553) that lies within the coiled-coil domain of ROCK II is responsible for its centrosomal localization (Ma et al., 2006), which differs from the Rho-binding domain (a.a. 979-1047).

RhoA- and RhoC-associated promotion of centrosome duplication is ROCK II-dependent

Like other small GTPases, Rho binds to and controls the activities of many proteins (reviewed in Bishop and Hall, 2000). Thus, it is possible that Rho may control centrosome duplication through multiple pathways, including the ROCK II pathway. To this end, we examined the abilities of the constitutively active L63 RhoA and RhoC mutants to drive centrosome duplication in cells silenced for ROCK II expression. As previously shown (Ma *et al.*, 2006), ROCK II could be transiently silenced in NIH3T3 cells to <10% of the normal expression level by siRNA (ROCK II RNAi cells) (Fig. 4A, second panel). ROCK II RNAi and control cells were transfected with constitutively active (L63) and dominant negative (N19) mutants of RhoA and RhoC (Fig. 4A, top panel). The transfected cells were then subjected to the centrosome re-duplication assay (Fig. 4B). Both RhoA-L63 and RhoC-L63 failed to promote centrosome duplication in the ROCK II RNAi cells, indicating that the centrosome duplication promoting activities of RhoA and RhoC requires the presence of ROCK II. However, the frequencies of centrosome re-duplication in the ROCK II RNAi cells transfected with RhoA-L63 as well as RhoC-L63 were small, but noticeably more than the control ROCK II RNAi cells (∼15% vs. ∼8%). Also, the frequencies of centrosome re-duplication in the ROCK II RNAi cells transfected with RhoA-N19 as well as RhoC-N19 dominant negative mutants were small, but noticeably less than the control ROCK II RNAi cells (∼5% vs. ∼8%). Although such differences may be due to either incomplete silencing of ROCK II in ROCK II RNAi cells or incomplete inhibition of endogenous Rho proteins by the dominant negative Rho mutants, it is also possible that there may be additional effector(s) of RhoA as well as RhoC other than ROCK II to drive centrosome duplication.

To test whether Rho controls centrosome duplication solely via targeting ROCK II or through multiple pathways, we examined the RhoA-L40 and RhoC-L40 mutants that have been reported to be defective for binding to and activating ROCK II (Sahai *et al.*, 1998) for their activities to promote centrosome duplication. Prior to this experiment, we analyzed the Rho-L40 mutant for their abilities to bind to and activate ROCK II. To test the ROCK II-binding activity of the L40 mutant, 293T cells were co-transfected with the ROCK II sequence (a.a. 600-1388) that contains the Rho-binding domain and FLAG-tagged RhoA-L40, RhoA-L63, or RhoA-L40/L63. FLAG-RhoA-L63 bound to ROCK II efficiently, while FLAG-RhoA-L40 and FLAG-RhoA-L40/L63 failed to do so (Supplemental Information Fig. 3), demonstrating that Rho-L40 mutant is indeed defective for binding to ROCK II. To test the L40 mutant for its ability to activate ROCK II, NIH3T3 cells were transfected with FLAG-tagged RhoA-N19, RhoA-L40, RhoA-L63, or RhoA-L40/L63. To examine the ROCK activation in the transfected cells, cells were stained for stress fibers with fluorescein-conjugated phalloidin. Expectedly, expression of RhoA-N19 resulted in reduction of stress fiber formation, while overexpression of RhoA-L63 resulted in strong stress fiber formation. In cells transfected with either RhoA-L40/L63 (Fig. 5A) or RhoA-L40 (data not shown), there was no difference in the stress fiber staining patterns with the vector-transfected control cells, demonstrating that the Rho-L40 mutant cannot activate ROCK. Confirming that the Rho-L40 mutant cannot bind to and activate ROCK II in our experimental systems, NIH3T3 cells were transfected with RhoA-L40 and RhoC-L40 as well as constitutively active forms of RhoA-L40 and RhoC-L40 (RhoA-L40/L63, RhoC-L40/L63, respectively). As positive and negative controls, constitutively active RhoA-L63

and RhoC-L63 mutants as well as vector plasmids were transfected. After confirming that all Rho mutants were expressed at comparable levels (Fig. 5B), the transfected cells were subjected to the centrosome re-duplication assay (Fig. 5C; representative immunostaining images are shown in Fig. 5D). RhoA-L40 and RhoC-L40 ROCK II-binding/activation mutants as well as The RhoA-L40/L63 and RhoC-L40/L63 double mutants failed to promote centrosome duplication, indicating that ROCK II is a primary target of RhoA and RhoC to promote centrosome duplication.

If ROCK II is truly a primary target of RhoA and RhoC for the initiation of centrosome duplication, the constitutively active ROCK II (CAT mutant), whose activity is independent of the Rho-binding, should be able to restore the ability to initiate centrosome duplication in cells silenced for either RhoA or RhoC. To test this possibility, NIH3T3 cells were transfected with the CAT mutant along with either RhoA or RhoC siRNA sequence. The transfected cells were then subjected to the centrosome re-duplication assay. As expected, silencing of RhoA and RhoC resulted in suppression of centrosome re-duplication. However, when the CAT mutant was co-expressed, cells silenced for either RhoA or RhoC re-duplicated centrosomes at frequencies similar to those transfected with the CAT mutant alone (Fig. 5E). Thus, the constitutively active ROCK II can fully restore the centrosome duplication potential in cells silenced for RhoA- as well as RhoC, further demonstrating that ROCK II is a primary effector of RhoA and RhoC to promote centrosome duplication.

Discussion

The centrosome/centriole duplication cycle is coupled with the DNA replication cycle, which is one of the important mechanisms that ensure centrosomes to duplicate only once in a single cell cycle. The coupling of these two events is at least in part achieved by the late G1 phase-specific activation of CDK2-cyclin E. CDK2-cyclin E targets a number of proteins, which leads to initiation of both centrosome duplication and DNA replication. We have previously shown that NPM/B23 is one of the targets of CDK2-cyclin E to initiate centrosome duplication (Okuda *et al.*, 2000; Tokuyama *et al.*, 2001). NPM/B23 phosphorylated by CDK2-cyclin E binds to and super-activates ROCK II, which is a critical event for the timely initiation of centrosome duplication (Ma *et al.*, 2006). Activation of ROCK II also requires the binding of Rho-GTPase to the Rho binding domain at C-terminus, which releases the kinase domain from the autoinhibitory domain. Although all Rho isoforms (RhoA, RhoB, and RhoC) are capable of binding to and activating ROCK II, we found that RhoA and RhoC, but not RhoB, participate in the regulation of centrosome duplication. For instance, knock-down of either RhoA or RhoC resulted in suppression of centrosome duplication, while knock-down of RhoB had no effect on centrosome duplication. The activity of Rho is regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), which facilitate the exchange of GDP to GTP, GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis to GDP, and GDP dissociating inhibitors (GDIs), which inhibit spontaneous GDP-GTP exchange of Rho (reviewed in Kjoller and Hall, 1999). The failure of RhoB to promote centrosome duplication is not due to the failure to be activated by these regulatory proteins, since expression of constitutively active form of RhoB, unlike that of RhoA and RhoC, fails to efficiently promote centrosome duplication. Instead, we found that constitutively active RhoA and RhoC localize to

centrosomes, while RhoB failed to do so. Because ROCK II localizes to centrosomes throughout the cell cycle, and drives centrosome duplication at centrosomes (Ma *et al.*, 2006), the ability of Rho to be recruited to centrosomes is expected to be required for controlling centrosome duplication. Thus, the failure of RhoB to control centrosome duplication is likely because of its inability to localize to centrosomes. Although our present studies show that RhoB is not involved in centrosome duplication during normal cell cycle or centrosome re-duplication in late G1 and S phases, our studies do not exclude the involvement of RhoB in centrosome amplification that occurs in late G2 phase. For instance, there is one study implicating RhoB in radiation-induced centrosome amplification, which is known to occur in late G2 phase of the cell cycle (Milia *et al.*, 2005).

In respect to the centrosome localization activity of Rho, we further found that GDP-bound inactive forms of RhoA and RhoC fail to localize to centrosomes or do so very inefficiently. However, this observation does not answer whether RhoA/C is recruited to centrosomes as a GDP-bound form or GTP-bound form. It is possible that RhoA/C may be recruited to centrosomes as a GDP-bound form, and GDP-GTP exchange may occur at centrosomes. In support of this possibility, some RhoGEFs such as Ect2 (Wolf *et al.*, 2006) and ARHGEF10 (Aoki *et al.*, 2009) have been shown to reside at centrosomes, indicating that GDP-GTP exchanges of Rho can occur at centrosomes.

The observation that depletion of RhoA alone and that of RhoC alone both resulted in suppression of centrosome duplication raises a question of whether there is any functional difference between RhoA and RhoC to control centrosome duplication. Our present studies indicate that ROCK II is a primary target of both RhoA and RhoC to control centrosome duplication. For instance, the ROCK II-binding/activation defective mutant RhoA and RhoC both failed to promote centrosome duplication. Based on the finding that introduction of constitutively active RhoC in the RhoA RNAi cells as well as introduction of constitutively active RhoA in the RhoC RNAi cells restores the centrosome duplication potential of the cells, it is possible that RhoA and RhoC may comprise the intracellular concentration of the total Rho proteins required for initiation of centrosome duplication. However, alternatively, although the primary target of both RhoA and RhoC is ROCK II, RhoA and RhoC may possess their own unique functions toward initiation of centrosome duplication besides ROCK II activation, and such unique function(s) of one isoform may be readily compensated by the overexpression/overactivation of the other isoform.

Our present findings put forward to several significant clinical implications. Overactivation of RhoA as well as RhoC is commonly found in human cancers (reviewed in Gómez del Pulgar et al., 2005; Ellenbroek and Collard, 2007; Vega and Ridley, 2008). We found that overexpression/overactivation of RhoA and RhoC promotes centrosome duplication via ROCK II. Moreover, stable expression of constitutively active forms of RhoA and RhoC results in a high frequency of centrosome amplification and chromosome instability (data not shown). Because the presence of amplified centrosomes destabilizes chromosomes, centrosome amplification is likely an important factor contributing to carcinogenesis associated with overexpression/overactivation of RhoA and RhoC. The other implication is in association with aberrant activation of receptor tyrosine kinases (RTKs) and carcinogenesis. The uncontrolled activation of RTKs is one of the most common features of

cancers. It has been recognized that oncogenic activation of many receptor tyrosine kinases leads to destabilization of chromosomes. However, this phenomenon has been belittled as an indirect consequence of the continuous firing of the cell cycle signaling. Because Rho is one of the immediate effectors of a wide variety of RTKs (reviewed in Kjoller and Hall, 1999), our present finding, in which over-activation of Rho leads to centrosome amplification via ROCK II, suggest that oncogenic activation of RTKs may be more directly involved in destabilization of chromosomes through generation of amplified centrosomes by continual activation of the Rho-ROCK II pathway. Consistently to this possibility, it has been shown that continual (oncogenic) activation of EGF receptor has been shown to promote centrosome duplication (Balczon *et al.*, 1996). Similarly, stable expression and continual activation of the Met hepatocyte growth factor (HGF) receptor in fibroblasts (*i.e.*, M114 cell line supplemented with excess HGF, ref. Shinomiya *et al.*, 2004) leads to centrosome amplification in a ROCK II-dependent manner (Supplemental Information: Fig. 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Rowland and B. Nepon-Sixt for technical assistance and core staff at the analytic microscopy core facility and molecular biology core facility in H. Lee Moffitt cancer center. We also thank Dr. Sebti for providing RhoB cDNA. This study is supported by National Institute of Health (CA90522 to K.F) and James & Esther King Biomedical Research Program (09KN-05-23139 to M.K).

References

Aoki T, Ueda S, Kataoka T, Satoh T. Regulation of mitotic spindle formation by the RhoA guanine nucleotide exchange factor ARHGEF10. BMC Cell Biol. 2009; 28:56. [PubMed: 19635168]

Balczon R, Bao L, Zimmer WE, Brown K, Zinkowski RP, Brinkley BR. Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. J Cell Biol. 1995; 130:105–115. [PubMed: 7790366]

Bishop AL, Hall A. Rho GTPases and their effector proteins. Biochem J. 2000; 348:241–255. [PubMed: 10816416]

Canguilhem B, Pradines A, Baudouin C, Boby C, Lajoie-Mazenc I, Charveron M, Favre G. RhoB protects human keratinocytes from UVB-induced apoptosis through epidermal growth factor receptor signaling. J Biol Chem. 2005; 280:43257–43263. [PubMed: 16278215]

Chen Z, Indjeian VB, McManus M, Wang L, Dynlacht BD. CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. Dev Cell. 2002; 3:339–350. [PubMed: 12361598]

Chevrier V, Piel M, Collomb N, Saoudi Y, Frank R, Paintrand M, Narumiya S, Bornens M, Job D. The Rho-associated protein kinase p160ROCK is required for centrosome positioning. J Cell Biol. 2002; 157:807–817. [PubMed: 12034773]

D′Assoro AB, Lingle WL, Salisbury JL. Centrosome amplification and the development of cancer. Oncogene. 2002; 40:6146–6153. [PubMed: 12214243]

Doxsey S. Re-evaluating centrosome function. Nature Rev Mol Cell Biol. 2001; 2:688–698. [PubMed: 11533726]

Ellenbroek SI, Collard JG. Rho GTPases: functions and association with cancer. Clin Exp Metastasis. 2007; 24:657–672. [PubMed: 18000759]

Fisk HA, Winey M. The mouse Mps1p-like kinase regulates centrosome duplication. Cell. 2001; 106:95–104. [PubMed: 11461705]

Fukasawa K. Centrosome amplification, chromosome instability and cancer development. Cancer Lett. 2005; 230:6–19. [PubMed: 16253756]

Fukasawa K. Oncogenes and tumour suppressors take on centrosomes. Nat Rev Cancer. 2007; 7:911–924. [PubMed: 18004399]

Gómez del Pulgar T, Benitah SA, Valerón PF, Espina C, Lacal JC. Rho GTPase expression in tumourigenesis: evidence for a significant link. Bioessays. 2005; 27:602–613. [PubMed: 15892119]

Hinchcliffe EH, Li C, Thompson EA, Maller JL, Sluder G. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science. 1999; 283:851–854. [PubMed: 9933170]

Hinchcliffe EH, Sluder G. Two for two: Cdk2 and its role in centrosome doubling. Oncogene. 2002; 21:6154–6160. [PubMed: 12214244]

Ho TT, Merajver SD, Lapière CM, Nusgens BV, Deroanne CF. RhoA-GDP regulates RhoB protein stability. Potential involvement of RhoGDIalpha. J Biol Chem. 2008; 283:21588–21598. [PubMed: 18524772]

Kabuyama Y, Litman ES, Templeton PD, Metzner SI, Witze ES, Argast GM, Langer SJ, Polvinen K, Shellman Y, Chan D, Shabb JB, Fitzpatrick JE, Resing KA, Sousa MC, Ahn NG. A mediator of Rho-dependent invasion moonlights as a methionine salvage enzyme. Mol Cell Proteomics. 2009; 8:2308–2320. [PubMed: 19620624]

Kjoller L, Hall A. Signalling to Rho GTPases. Exp Cell Res. 1999; 253:166–179. [PubMed: 10579921]

Lacey KR, Jackson PK, Stearns T. Cyclin-dependent kinase control of centrosome duplication. Proc Natl Acad Sci USA. 1999; 96:2817–2822. [PubMed: 10077594]

Ma Z, Kanai M, Kawamura K, Kaibuchi K, Ye K, Fukasawa K. Interaction between ROCK II and nucleophosmin/B23 in the regulation of centrosome duplication. Mol Cell Biol. 2006; 26:9016–9034. [PubMed: 17015463]

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 1996; 15:2208–2216. [PubMed: 8641286]

Matsumoto Y, Hayashi K, Nishida E. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Curr Biol. 1999; 9:429–432. [PubMed: 10226033]

Milia J, Teyssier F, Dalenc F, Ader I, Delmas C, Pradines A, Lajoie-Mazenc I, Baron R, Bonnet J, Cohen-Jonathan E, Favre G, Toulas C. Farnesylated RhoB inhibits radiation-induced mitotic cell death and controls radiation-induced centrosome overduplication. Cell Death Differ. 2005; 12:492–501. [PubMed: 15776002]

Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol. 1997; 13:261–291. [PubMed: 9442875]

Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett. 1996; 392:189–193. [PubMed: 8772201]

Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, Knudsen ES, Hofmann IA, Snyder JD, Bove KE, Fukasawa K. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell. 2000; 103:127–140. [PubMed: 11051553]

Reed SI. Control of the G1/S transition. Cancer Surv. 1997; 29:7–23. [PubMed: 9338094]

Sahai E, Alberts AS, Treisman R. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. EMBO J. 1998; 17:1350–1361. [PubMed: 9482732]

Salisbury JL. A mechanistic view on the evolutionary origin for centrin-based control of centriole duplication. J Cell Physiol. 213:420–428. [PubMed: 17694534]

Shinomiya N, Gao CF, Xie Q, Gustafson M, Waters DJ, Zhang YW, Vande Woude GF. RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. Cancer Res. 2004; 64:7962–7970. [PubMed: 15520203]

Simpson KJ, Dugan AS, Mercurio AM. Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. Cancer Res. 2004; 64:8694–8701. [PubMed: 15574779]

Tarapore P, Horn HF, Tokuyama Y, Fukasawa K. Direct regulation of the centrosome duplication cycle by the p53-p21Waf1/Cip1 pathway. Oncogene. 2001; 20:3173–3184. [PubMed: 11423967]

Tarapore P, Okuda M, Fukasawa K. A mammalian in vitro centriole duplication system: evidence for involvement of CDK2/cyclin E and nucleophosmin/B23 in centrosome duplication. Cell Cycle. 2002; 1:75–81. [PubMed: 12429912]

Tokuyama Y, Horn HF, Kawamura K, Tarapore P, Fukasawa K. Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. J Biol Chem. 2001; 276:21529–21537. [PubMed: 11278991]

Vega FM, Ridley AJ. RhoGTPases in cancer cell biology. FEBS lett. 2008; 582:2093–2101. [PubMed: 18460342]

Wang HR, Zhang Y, Ozdamar B, Ogunjimi AA, Alexandrova E, Thomsen GH, Wrana JL. Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science. 2003; 302:1775–1779. [PubMed: 14657501]

Wang L, Yang L, Luo Y, Zheng Y. A novel strategy for specifically down-regulating individual Rho GTPase activity in tumor cells. J Biol Chem. 2003; 278:44617–44625. [PubMed: 12939257]

Wheeler AP, Ridley AJ. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res. 2004; 301:43–49. [PubMed: 15501444]

Wolf A, Keil R, Götzl O, Mun A, Schwarze K, Lederer M, Hüttelmaier S, Hatzfeld M. The armadillo protein p0071 regulates Rho signaling during cytokinesis. Nat Cell Biol. 2006; 8:1432–1440. [PubMed: 17115030]

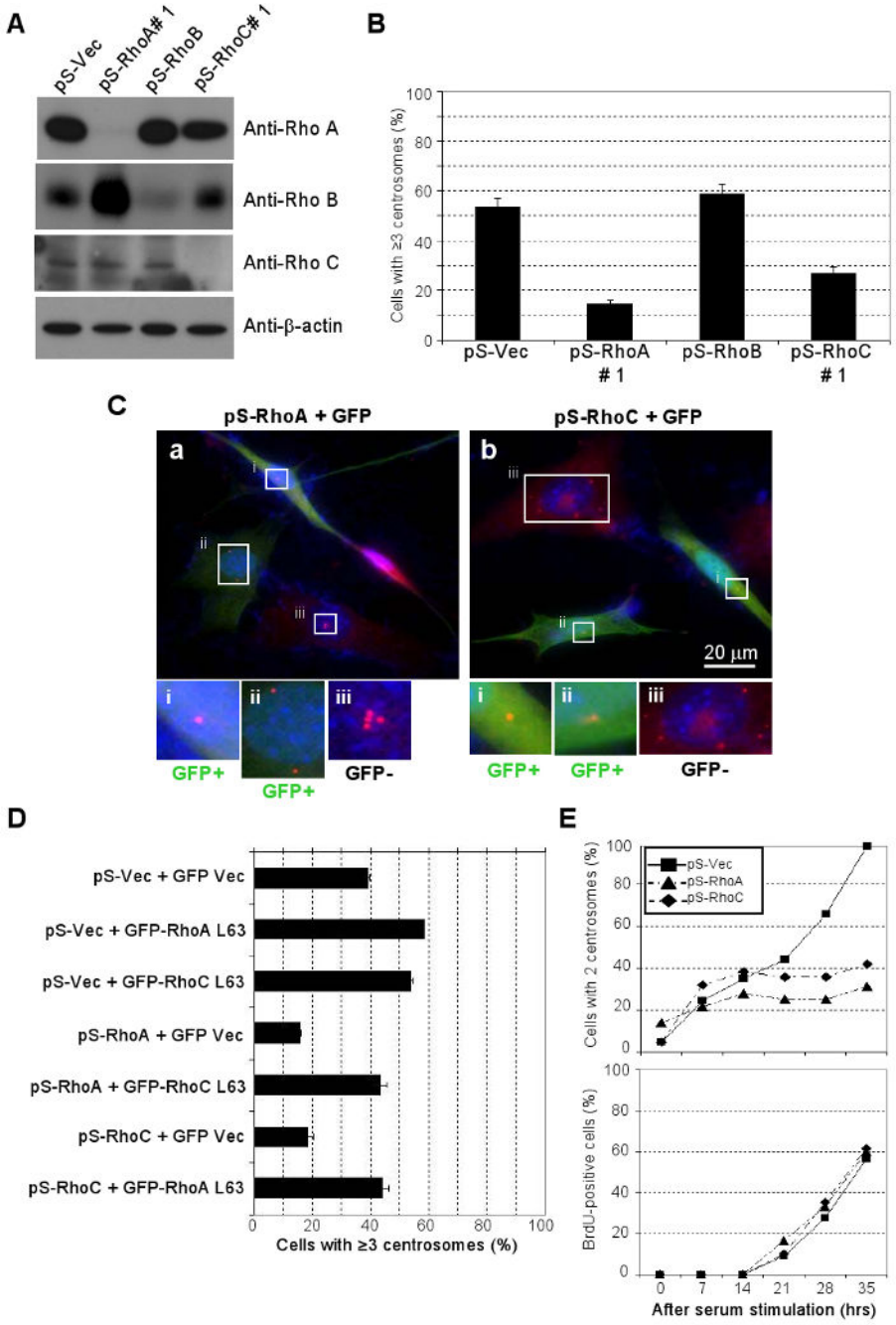

Fig. 1. Depletion of RhoA and RhoC, but not RhoB, suppresses centrosome duplication

NIH3T3 cells were co-transfected with a pSuper (pS) plasmid containing the siRNA sequences specific for each Rho isoform and a GFP-plasmid containing a neomycin-resistance gene. As a control, a pS plasmid containing a randomized siRNA sequence was transfected. After G418 selection, the surviving cells were pooled. The lysates prepared from the transfected cells were immunoblotted with anti-RhoA, anti-RhoB, and anti-Rho C antibodies (A). The transfected cells were exposed to Aph for 48 h, and examined for the centrosome profiles by immunostaining with anti-γ-tubulin and anti-GFP antibodies. DNA

was stained with DAPI. The number of centrosomes per cell was scored in the GFP-positive cells, and the results are shown in the graph (B) as the average ± standard error from three experiments. For each experiment, >200 cells were examined. The representative immunostaining images of the GFP-positive and neighboring GFP-negative cells transfected with RhoA (#1) and RhoC siRNAs (#1) are shown in (C). The magnified images of the indicated areas (i-iii) are shown in the bottom panels. NIH3T3 cells were co-transfected with a pSuper plasmid containing the siRNA sequences specific for RhoA (#1) or RhoC (#1) and a plasmid containing either GFP-RhoA-L63 or GFP-RhoC-L63. As a control, a pSuper plasmid with a randomized siRNA sequence and a GFP vector plasmid were co-transfected. The transfected cells were subjected to the centrosome re-duplication assay (Aph for 30 h). The number of centrosomes per cell was scored in the GFP-positive cells, and the results are shown in the graph (D) as the average ± standard error from three experiments. MSFs were serum-starved by 0.2% FBS for 24 hrs, and co-transfected with a pS plasmid containing the siRNA sequence specific for each Rho isoform and GFP as a marker. After total 48 hr serum starvation, cells were serum-stimulated by 20% FBS in the presence of BrdU. The cells were then monitored every 7 hr for the period of 35 hrs for the rates of centrosome duplication and BrdU-incorporation as described previously (Ma et al., 2006), and the results were shown in the graph (E). pS-vector control: square (■), pS-RhoA (#1): triangle (▲), and pS-RhoC (#1): diamond (◆).

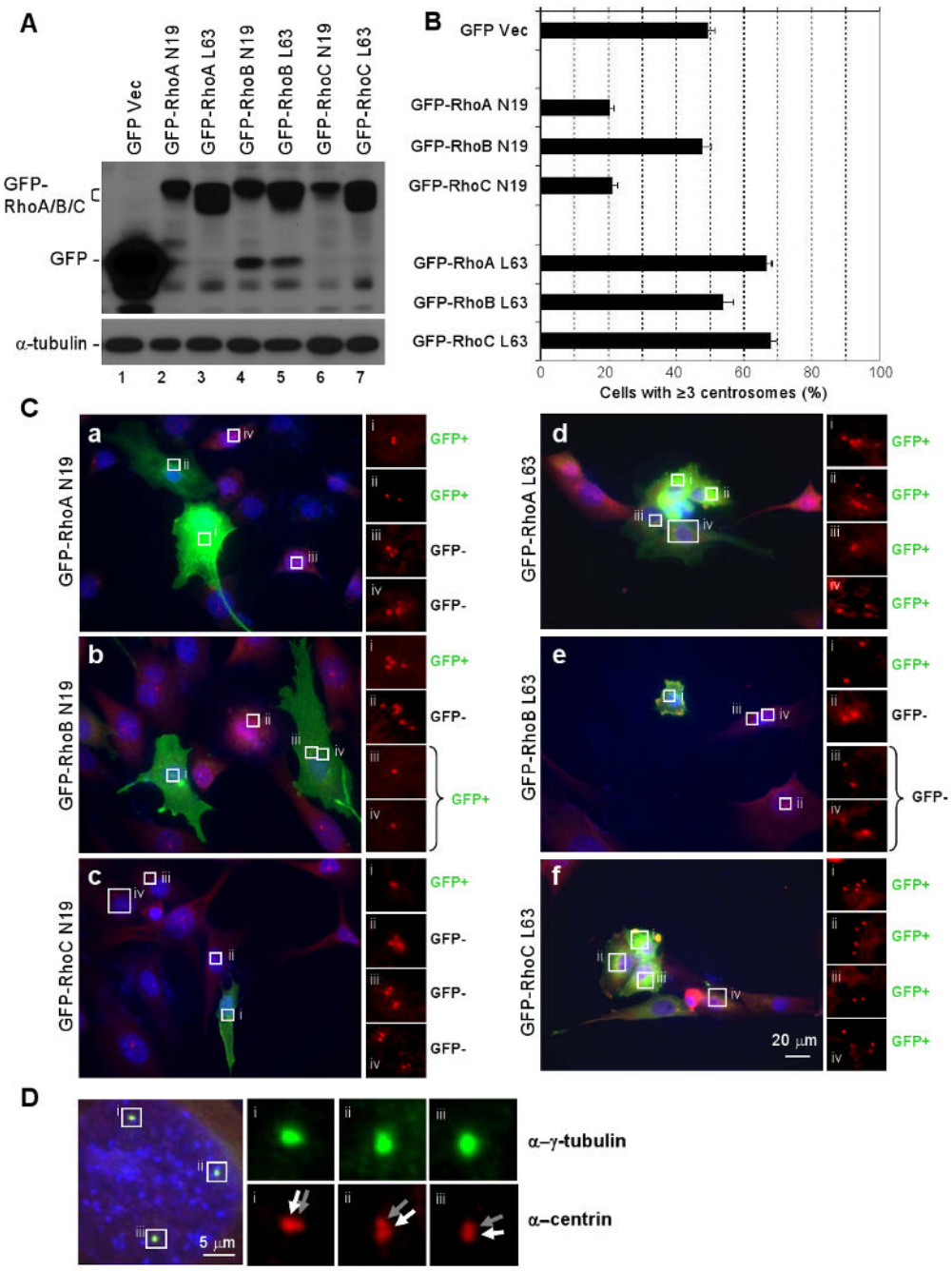

Fig. 2. Effects of the expression of dominant negative and constitutively active mutant Rho proteins on centrosome duplication

NIH3T3 cells were transfected with a plasmid encoding GFP-tagged RhoA-N19, RhoB-N19, RhoC-N19, RhoA-L63, RhoB-L63 or RhoC-L63. As a control, a GFP vector plasmid was transfected. The lysates prepared from the transfected cells were immunoblotted with anti-GFP antibody (A). The transfected cells were subjected to the centrosome re-duplication assay (Aph for 48 h). After immunostaining with anti-γ-tubulin and anti-GFP antibodies and counter-staining DNA with DAPI, the number of centrosomes per cell was scored in the GFP-positive cells, and the results are shown in the graph (B) as the average ±

standard error from three experiments. The representative immunostaining images of the GFP-positive and neighboring GFP-negative cells are shown in (C). The magnified images of the indicated areas (i-iv) are shown at the right of each panel. NIH3T3 cells were transfected with FLAG-RhoA L63 and puromycin gene (plasmid DNA ratio is 20:1) and after 3 days drug selection, centrosome re-duplication assay was performed. To show the centrosome integrity, the amplified centrosomes were co-immunostained with anti-γ-tubulin (green) and anti-centrin (red) antibodies (D). Each single γ-tubulin spot contains a pair of centrioles. The magnified images of the indicated areas (i-iii) are shown at the right of each panel.

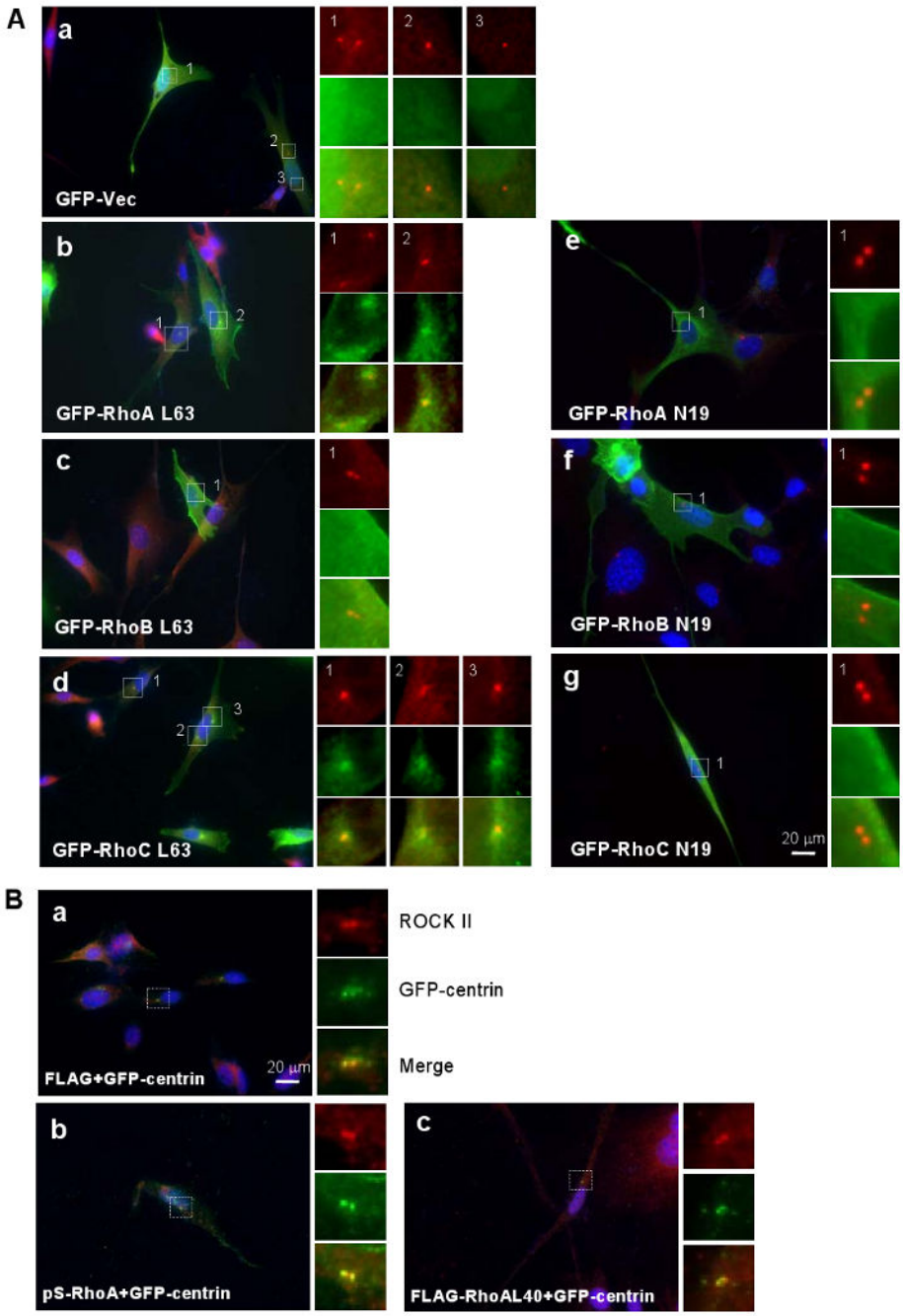

Fig.3. Constitutively active mutants of RhoA and RhoC, but not RhoB, localize to centrosomes independently from ROCK II

(A) NIH3T3 cells were transfected with a plasmid containing GFP-tagged constitutively active (L63) and dominant negative (N19) of RhoA, RhoB or RhoC, and immunostained with anti-γ-tubulin (red) and anti-GFP antibodies (green), and then counterstained for DNA with DAPI (blue). The magnified images of the indicated areas in panels a-g are shown at the right side; γ-tubulin: top panels (red), GFP: middle panels (green), overlay: bottom panels in each set. (B) NIH3T3 cells were transfected with FLAG-vector, pSuper-RhoA or FLAG-tagged L40 of RhoA. GFP-centrin was co-transfected as a transfection as well as a

centriole marker (10:1 ratio). The transfected cells were briefly extracted prior to fixation (see Materials and Methods), and subjected to immunostaining with anti-GFP and anti-ROCK II antibodies. The magnified images of the indicated areas in panels a-c are shown on the right. ROCK II: top panels (red), GFP (GFP-centrin): middle panels (green), overlay: bottom panels in each set.

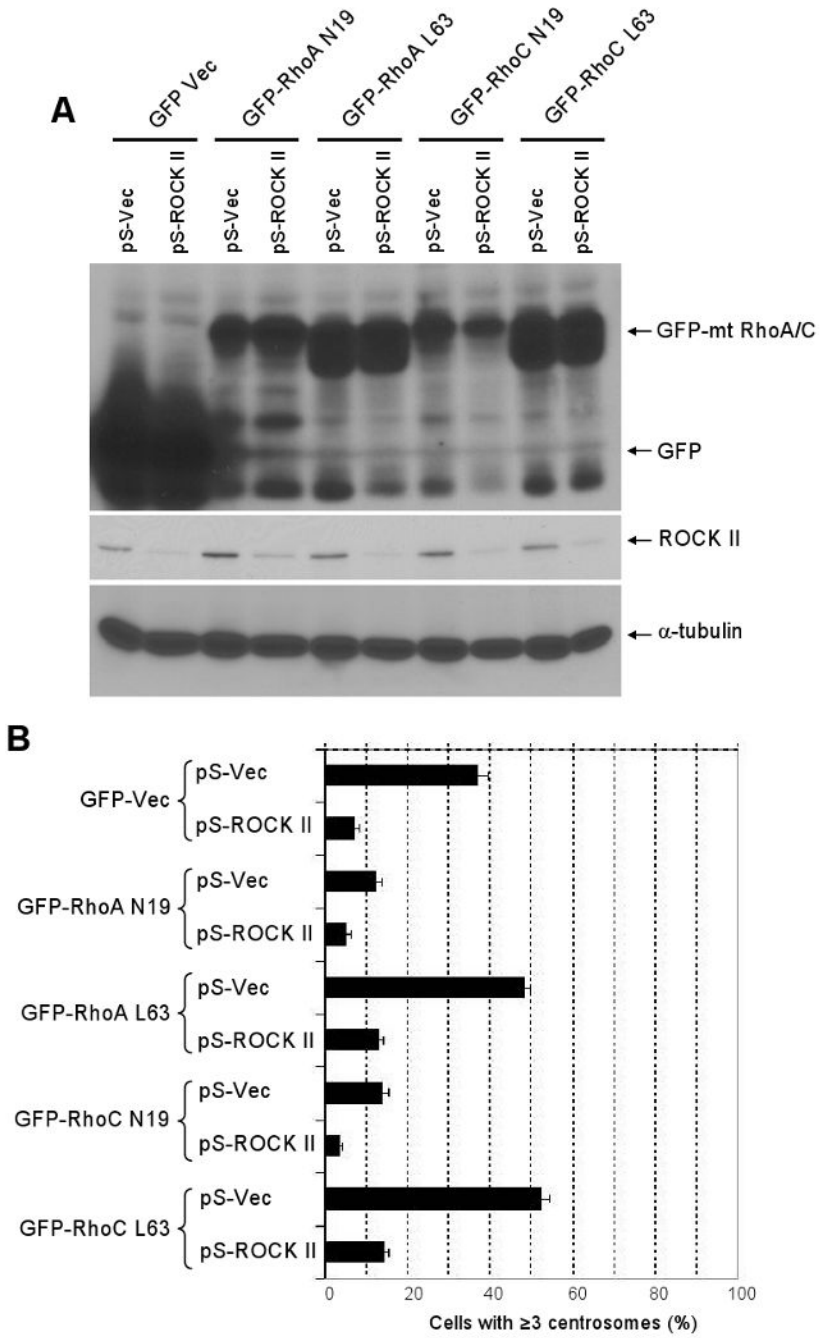

Fig. 4. The centrosome duplication promoting activities of RhoA and RhoC depend on the presence of ROCK II

NIH3T3 cells were transfected with ROCK II siRNA (pS-ROCK II) along with a puromycin-resistant plasmid (with 10:1 ratio). As a control, a pS plasmid containing a randomized siRNA sequence was transfected. After puromycin selection for 3 days, the surviving cells were pooled, and transfected with GFP-tagged RhoA-N19, RhoA-L63, RhoC-N19 and Rho-L63. As a control, a GFP-vector plasmid was transfected. The lysates prepared from the transfected cells were immunoblotted with anti-GFP and anti-ROCK II antibodies (A). The transfected cells were also subjected to the centrosome re-duplication

assay (Aph exposure; 24h). The centrosome profiles were scored for the GFP-positive cells, and the results are shown in the graph (B) as the average ± standard error from three experiments.

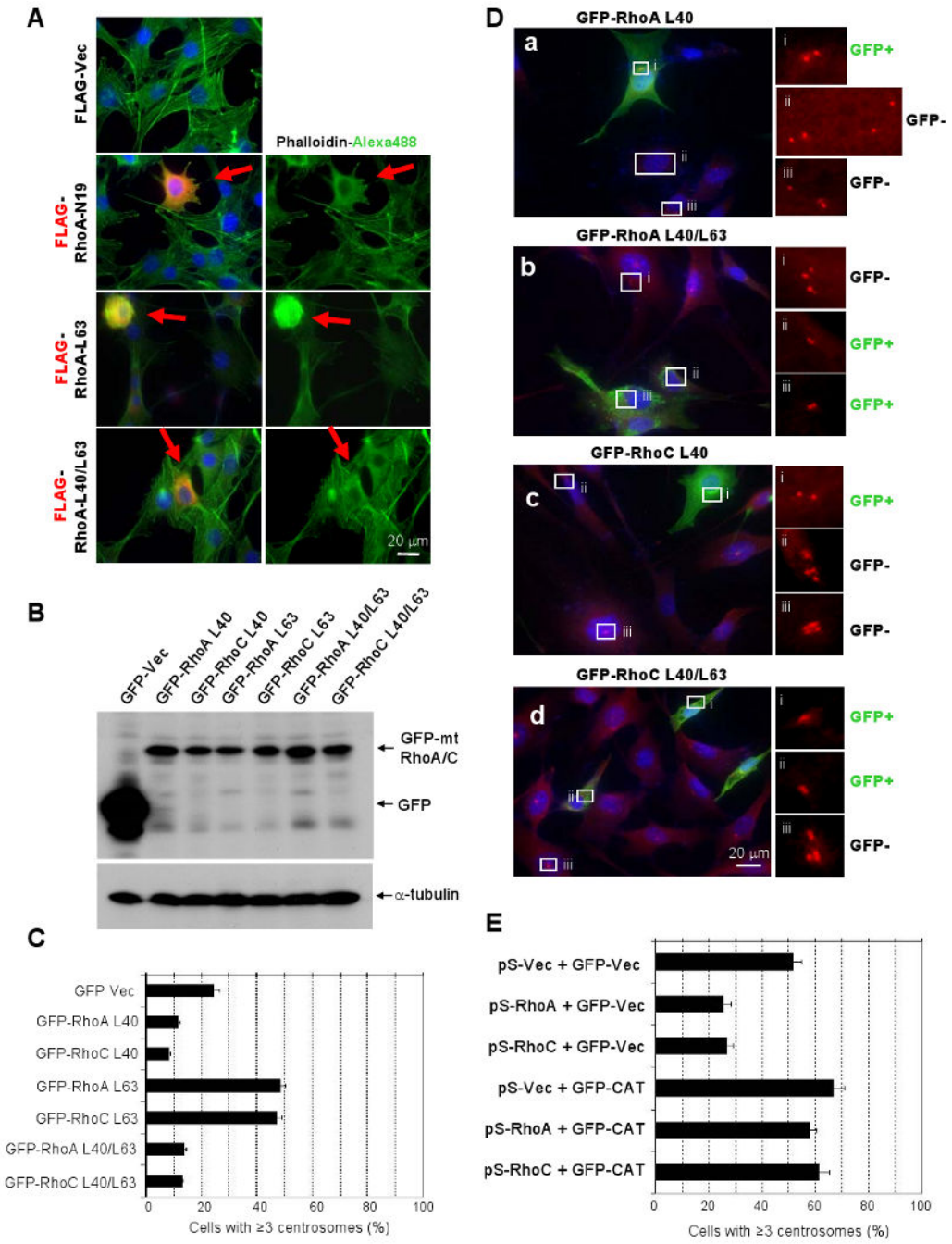

Fig. 5. Loss of the centrosome duplication promoting activity in the RhoA and RhoC mutant defective for ROCK II recognition

(A) For the stress fiber formation assay, NIH 3T3 cells were transfected with FLAG-vector, FLAG-RhoA-N19 (negative control), FLAG-RhoA-L63 (positive control), or FLAG-RhoA-L40/L63, and the transfectants were immunostained with anti-FLAG antibody. Actin stress fibers were stained with phalloidin-alexa488. Red arrows indicate FLAG-Rho expressing cells. (B) NIH3T3 cells were transfected with GFP-tagged RhoA- and RhoC-L40 (ROCK II-binding/activation defective mutants), RhoA- and RhoC-L63 (constitutively active mutants), and RhoA- and RhoC-L40/L63 (constitutively active ROCK II-binding/activation double

mutants), and the lysates prepared from the transfected cells were immunoblotted with anti-GFP antibody (B). The transfected cells were also subjected to the centrosome re-duplication assay (Aph exposure; 20h). The centrosome profiles were scored for the GFP-positive cells, and the results are shown in the graph (C) as the average ± standard error from three experiments. The representative immunostaining images of the GFP-positive and neighboring GFP-negative cells are shown in (D). The magnified images of the indicated areas (i-iii) are shown at the right of each panel. (E) NIH3T3 cells were transfected with a pSuper plasmid containing a randomized siRNA, RhoA siRNA or RhoC siRNA sequence along with either a GFP-vector plasmid or GFP-CAT (Rho-independent constitutively active ROCK II mutant) at 10:1 ratio. The transfected cells were then exposed to Aph for 48 h, and examined for the centrosome profiles: the number of centrosomes per cell was scored in the GFP-positive cells, and the results are shown in the graph as the average ± standard error from three experiments.