The RhoGAP ARHGAP19 controls cytokinesis and chromosome segregation in T lymphocytes

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Summary

Small GTP-binding proteins of the Rho family orchestrate the cytoskeleton remodeling events required for cell division. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) promote cycling of Rho GTPases between the active GTP-bound and the inactive GDP-bound conformations. We report that ARHGAP19, a previously uncharacterized protein, is predominantly expressed in hematopoietic cells and is a critical actor of T lymphocyte division. Overexpression of ARHGAP19 in lymphocytes delays cell elongation and cytokinesis. Conversely, silencing of ARHGAP19 or expression of a GAP-deficient mutant induces precocious mitotic cell elongation and cleavage furrow ingestion, as well as excessive blebbing. In relation with these phenotypes, we show that ARHGAP19 acts as a GAP for RhoA, and controls Citron and Myosin II recruitment to the plasma membrane of mitotic lymphocytes as well as Rock2-mediated phosphorylation of Vimentin, a critical determinant in stiffness and shape of lymphocytes. In addition to its effects on cell shape changes, silencing of ARHGAP19 in lymphocytes also impairs chromosome segregation.
Introduction

Cytokinesis, the process whereby one dividing cell splits its cytoplasm into two equal parts, involves drastic cell shape changes, especially for adherent cells that become spherical when entering mitosis and maintain contact with the extracellular matrix only through retraction fibers. For both adherent cells and cells dividing in suspension such as lymphocytes, an approximately 1.5-fold elongation of the cell in anaphase precedes and accompanies ingression of the cleavage furrow that pinches off the cytoplasm at the cell equator. At later stages, again, morphological changes specific to adherent cells occur, since daughter cells separated by an intercellular bridge re-adhere to the extracellular matrix, while cells dividing in suspension conserve their shape until the intercellular bridge is severed, hours later. Despite these differences in morphological changes during division of adherent and suspension cells, the molecular mechanisms regulating cytokinesis have been primarily studied using adherent cells (Kittler et al., 2007; Moffat et al., 2006; Neumann et al., 2006; Neumann et al., 2010) and little is known on how lymphocyte division is controlled.

In adherent cell cytokinesis, activation of the small GTPase RhoA by GEFs such as Ect2, GEF-H1 and MyoGEF/PLEKHG6 increases cortical rigidity required for cell rounding in early mitosis and controls the actomyosin contractile ring during cleavage furrow ingression (Birkenfeld et al., 2007; Green et al., 2012; Jaffe and Hall, 2005; Kamijo et al., 2006; Maddox and Burridge, 2003; Tatsumoto et al., 1999; Wu et al., 2006). While much attention has been given to the mechanisms by which Rho is activated during cytokinesis, it is still unclear how and when RhoA is inactivated. It remains to be determined whether GAP-dependent downregulation of RhoA activity occurs throughout cytokinesis, to maintain an adequate level of active RhoA and/or to stimulate the RhoA GTPase flux (Miller et al., 2008), or much later in the division process. Moreover, only two GAP proteins limiting activity of Rho family GTPases have been shown to be involved in cytokinesis. Experimental overexpression of p190A RhoGAP in adherent cells has been reported to decrease RhoA-dependent cleavage furrow ingression (Su et al., 2009) but the exact role of the endogenous protein has not been demonstrated. In adherent cells, RacGAP1/MgcRacGAP is paradoxically required for RhoA activation rather than for its inactivation (Loria et al., 2012) and was recently shown to act during anaphase to inhibit Rac1-dependent cell spreading and adhesion (Bastos et al., 2012). In contrast, the GAP activity of RacGAP1 is dispensable for cytokinesis in DT40 B lymphocytes (Yamada et al., 2006).
Here we describe a novel Rho GAP, ARHGAP19, predominantly expressed in cells of the hematopoietic lineage, which proved critical for cytokinesis in T lymphocytes.
Results

**Microarray analysis of Rho GTPase pathways in human leukemia samples**

During the course of experiments aimed at better understanding the role of Rho GTPases in hematopoietic cells, we analyzed relative expression levels of 300 genes of the Rho GTPase pathways (GEFs, GAPs, effectors and Rho GTPases) in 100 samples of human T-cell acute lymphoblastic leukemia whose transcriptome had previously been characterized (Soulier et al., 2005). Two-dimensional clustering brought together, on one hand, clinical samples displaying similar “Rho” gene expression profiles and, on the other hand, genes of the Rho pathway whose expression levels co-vary across samples (Fig. 1A).

One of the gene clusters thus identified includes Ect2, RacGAP1, Citron (three proteins with established roles in cytokinesis (Chalamalasetty et al., 2006)), Net1 (an oncogene that activates RhoA (Chan et al., 1996)), as well as ARHGAP11A and ARHGAP19, two proteins with unknown functions (Fig. 1B). Among the 36 genes of the global transcriptome that co-vary the most with this cluster, 32 code for proteins whose role in cell division is already established (Fig. 1C). This enrichment in genes coding for proteins involved in cell division suggested that ARHGAP19 and ARHGAP11A might, alike Ect2, RacGAP1 and Citron, participate in mitosis/cytokinesis control. We undertook to test this hypothesis, and focused our study on ARHGAP19 as, with the noticeable exception of germ cells, it is expressed predominantly in cells of hematopoietic origin (Fig. S1 in supplementary material).

**ARHGAP19 expression levels fluctuate during T lymphocyte cell cycle**

Variations in ARHGAP19 levels during progression through IL-2-induced cell cycling of Kit225 T lymphocytes were monitored by RT-PCR. mRNA coding for ARHGAP19, RacGAP1, Ect2 and Net1 are induced together with mRNA coding for Cyclin A2, during the S-phase of cell cycle (Fig. 1D and (Seguin et al., 2009)). Alike RacGAP1, Ect2 and Net1, the ARHGAP19 protein is undetectable in G1, appears during the S/G2 phases, peaks at mitosis, and then drops at the beginning of the next cell cycle (Fig. 1E). The correlative variations in expression of ARHGAP19, Ect2 and RacGAP1, with maximal levels during mitosis, suggested that these proteins may operate, sequentially or in concert, to regulate aspects of a shared cell function.

**ARHGAP19 shuttles between nucleus, cytoplasm and cell cortex**
We next investigated ARHGAP19 localization during cell cycle. Consistent with our finding that ARHGAP19 expression is regulated during cell-cycle, many cells in interphase were not stained by the anti-ARHGAP19 antibody. When present, staining was primarily detected in the cell nuclei (Fig. 2A). Subcellular localization of ARHGAP19 varied during mitosis (Fig. 2B). In prophase and metaphase, ARHGAP19 was dispersed in the cytoplasm. In early anaphase ARHGAP19 was enriched all along the cell periphery, whereas at later stages and until the end of cell division ARHGAP19 was concentrated at the cortical region of the ingressing cleavage furrow.

Experimental manipulations of ARHGAP19 level or activity affect time of cell elongation and cleavage furrow formation, and impact on chromosome segregation

To investigate the role of ARHGAP19 in lymphocyte division, we engineered populations of Kit225 lymphocytes in which expression of ARHGAP19 was either increased through transfection with plasmids allowing doxycyclin-induced expression of the GFP-ARHGAP19 fusion protein, or silenced through transfection with shRNA constructs (Fig. 3A). Time-lapse microscopy experiments revealed that manipulation of ARHGAP19 levels impact on two aspects of cell division: time of cell shape changes (cell elongation and cleavage furrow formation) and chromosome segregation (Fig. 3B-G and Videos 1-4 in supplementary material).

Silencing or overexpression of ARHGAP19 induced mirror effects on morphological changes and mitotic blebbing. In control cells, elongation and furrow ingression occurred 3 and 4 minutes after anaphase onset, respectively. In cells overexpressing ARHGAP19, these two events were postponed to 7 and 8 minutes after anaphase onset, respectively, and occurrence of mitotic blebs was abolished. Despite the significant delays in cell elongation and cleavage furrow ingestion, most of these cells successfully completed mitosis. In contrast, ARHGAP19-depleted mitotic lymphocytes displayed signs of cortical hypercontractility, such as excessive blebbing. About half of these cells started elongating earlier than control cells, on average 2 minutes before anaphase onset. Cleavage furrow ingression also occurred earlier than in control cells, only 2 minutes after anaphase onset. Of note, about 44% of the ARHGAP19-deficient lymphocytes could not be taken into account in these quantitative analyses of time of cell elongation or of cleavage furrow ingression, because of the occurrence of chromosome mis-segregation events that made difficult determination of the exact time of anaphase onset. In that severe defects in chromosome segregation were often
preceded by extensive cell shape remodeling in early mitosis, the morphological phenotypes induced by ARHGAP19-depletion are likely underestimated in the quantifications shown in Fig. 3F and 3G.

ARHGAP19 silencing affected chromosome segregation in 43.6% of the ARHGAP19-deficient lymphocytes: at the time of cleavage furrow contraction, chromosomes had not fully segregated away from the furrow area, and lagging DNA persisted in the inter-cellular bridge (Fig. 3D, middle and lower panels). Occasionally, cytokinesis reversion occurred (possibly due to lagging DNA-induced activation of the NoCut pathway (Mendoza et al., 2009; Norden et al., 2006; Steigemann et al., 2009)), leading to cell binucleation. Similar phenotypes were obtained when ARHGAP19 was silenced in Jurkat lymphocytes (Fig. S2 in supplementary material).

Because manipulation of ARHGAP19 levels impacted on both timing of morphological changes and chromosome segregation, the question arose as to whether these two aspects were mechanistically linked or instead could occur independently. To ascertain that, indeed, ARHGAP19 regulates cell shape in early mitosis, lymphocytes were treated with nocodazole, which blocks mitosis progression at the prometaphase stage. Anti-phospho-H3 staining was performed to ensure that experimental variations in ARHGAP19 levels did not affect the spindle assembly checkpoint and that lymphocytes from all the nocodazole-treated populations were properly arrested in mitosis (Fig. 4A). More than 60% of the ARHGAP19-depleted lymphocytes became elongated and adopted improbable shapes. In contrast, 90% of the ARHGAP19-overexpressing lymphocytes stayed round (Fig. 4A and B). Control cells showed an intermediate behavior. This result confirmed that manipulation of ARHGAP19 levels impacts on cell shape during early mitosis, and indicated that this aspect of the phenotype does not rely on microtubule dynamics or chromosome segregation. Demonstrating that chromosome mis-segregation in ARHGAP19-depleted lymphocytes could occur independently of earlier abnormalities in morphological changes proved more challenging. Although extensive cell shape remodeling in early mitosis was often followed by severe defects in chromosome segregation, the extents of these two aspects of the phenotypes were not always correlated. For example, despite the ARHGAP19-depleted lymphocyte shown in the middle panel of Figure 3D elongated long before anaphase onset, chromosome plates efficiently segregated toward the cell poles (although some lagging DNA was detectable). In contrast, the ARHGAP19-depleted lymphocyte shown in the bottom panel of Figure 3D elongated only after anaphase onset, and nevertheless displayed much worse
defects in chromosome segregation. Thus, each of the two aspects of the phenotype is likely a
direct consequence of manipulation of ARHGAP19 levels, and can occur independently of
each other.

To investigate whether ARHGAP19 functions depended on its putative GAP activity,
we mutated the predicted catalytic arginine residue (R143) of the GAP domain. 34.3% of
GFP-ARHGAP19 Rmut-expressing Kit225 cells divided without noticeable defects (Upper
panels of Fig. 3E). The remaining 65.7% of these cells displayed mitotic abnormalities.
Indeed, cells often became ovoid as soon as the metaphase stage and defective chromosome
segregation at the time of cleavage furrow ingression was frequently observed (Middle and
lower panels of Fig. 3E). Expression of the Rmut form of GFP-ARHGAP19 also induced
major blebbing and/or additional cleavage furrows, and daughter cells often kept wiggling
extensively for several hours following cytokinesis. Thus, expression of the Rmut form of
GFP-ARHGAP19 did not reproduce the effects triggered by the WT, which demonstrates that
the phenotypes induced by the WT rely on GAP domain integrity. Instead, the Rmut form
triggered opposite effects, reminiscent of those observed in the two independent populations
of ShRNA-transfected lymphocytes, suggesting that it acts as a dominant negative.

ARHGAP19 displays GAP activity on RhoA, but not Rac1 or CDC42

That the mitotic phenotypes triggered by ARHGAP19 overexpression depended upon
the arginine 143 of the GAP domain suggested that effects of ARHGAP19 on cytokinesis
occur through regulation of one or several GTPases. By performing a GAP assay in vitro, we
found that ARHGAP19 WT is able to stimulate the GTP hydrolysis catalyzed by RhoA, but
not by Rac1 or CDC42 (Fig. 5A). Thus, ARHGAP19 can function as a GAP protein for
RhoA. As expected, the R143A mutant of ARHGAP19 displayed no GAP activity.

We obtained additional evidence that ARHGAP19 can regulate RhoA in vivo. A
cytoplasmic mutant of GFP-ARHGAP19, truncated of its C-terminal region that contains two
potential bi-partite NLS (Fig. S3 in supplementary material), was expressed in HeLa cells, in
which endogenous ARHGAP19 protein is hardly detectable (as assessed by RT-PCR analysis
of mRNA expression or immunoprecipitation / Western Blot experiments performed in
conditions similar to those used with lymphocytes in Fig. 3A). Expression of GFP-
ARHGAP19-ΔCter decreased cellular levels of active RhoA (but not Rac1 or CDC42)
assessed through pull-down experiments using unsynchronized cells (Fig. 5B), decreased
stress fibers in interphasic cells (an event known to lie downstream of RhoA activity) (Fig.
5C), and impaired the RhoA-dependent cell rounding (Fig. 5D) that normally occurs at the
beginning of mitosis of adherent cells (Maddox and Burridge, 2003). As expected, all these
effects depended upon arginine 143 of the GAP domain of ARHGAP19.

Experimental manipulations of ARHGAP19 level or activity affect recruitment of RhoA,
Citron and Myosin II at cell equator, and regulate Rock2-mediated phosphorylation of
Vimentin in early mitosis

To investigate the molecular mechanisms through which ARHGAP19 controls
lymphocyte division, we assessed effects of manipulation of ARHGAP19 levels or activity on
the subcellular localizations of RhoA (the direct target of ARHGAP19), Citron and Rock
(known effector kinases of RhoA involved in cytokinesis (D’Avino et al., 2004; Gruneberg et
al., 2006; Kosako et al., 2000; Madaule et al., 1998)), and Myosin IIA (a protein involved in
cytoskeleton contractility and whose membrane recruitment during cytokinesis is dependent
on Rock- and/or Citron-mediated phosphorylation of the Myosin Light Chain subunit (Uehara
et al., 2010)).

In control lymphocytes, RhoA, Citron and Myosin IIA were recruited to the equatorial
membrane at anaphase onset and concentrated in the ingressing cleavage furrow afterward
(Fig. 6A-D), as reported in other cell types. Overexpression of ARHGAP19 WT drastically
decreased membrane recruitment of RhoA, Citron and Myosin IIA (Fig. 6A-D). Conversely,
silencing of ARHGAP19, or expression of its dominant-negative R143A mutant, clearly
enhanced membrane recruitment of RhoA, Citron and Myosin IIA in about 50% of the cells at
the prophase and metaphase stages (Fig. 6A-D). Such increase may also occur to some extent
at later mitotic stages, although staining quantification did not reveal statistical differences.

Staining of lymphocytes with the anti-phospho-S1366 Rock2 antibody that recognizes
specifically the activated, autophosphorylated form of Rock2 (Chuang et al., 2012) revealed
its striking localization on a cage-like subcellular structure (Fig. 7A). This structure is
reminiscent of that formed in lymphocytes by the Vimentin intermediate filaments (Brown et
al., 2001), which can be phosphorylated by Rock (Goto et al., 1998). We observed that active
Rock2 (stained in green) and pS71-Vimentin (stained in red) globally co-localized (Fig. 7A),
although intensity variations of the two signals did not fully correlate (Fig. 7B), making it
unlikely that the anti-active Rock2 cross-reacted with phosphorylated vimentin. In addition,
reciprocal IP/WB experiments (not shown) confirmed that these two antibodies did not cross-
react. Global variations in levels of active Rock2 and pS71-Vimentin correlated during
mitosis progression (Fig. 7A). Indeed, in control lymphocytes, active Rock2 and pS71 Vimentin were readily detectable on the cage at the prophase and metaphase stages, and in the intercellular bridges separating daughter cells in telophase. ARHGAP19 WT overexpression drastically decreased levels of active Rock2 and pS71 Vimentin in prophase and metaphase. Conversely, levels of active Rock2 and pS71 Vimentin were increased by silencing of ARHGAP19 or expression of its dominant-negative R143A mutant at the prophase and metaphase stages, and were still readily detectable in anaphase. Thus, as compared to control cells, ARHGAP19-deficient lymphocytes displayed increased, and possibly prolonged, levels of active Rock2 and pS71 Vimentin. Phosphorylation of Vimentin has been described to increase flexibility of the cage subtending the plasma membrane (Inagaki et al., 1987), which is required for large morphological changes to occur in lymphocytes (Paulin-Levasseur and Brown, 1987). Thus, ARHGAP19 ability to regulate the RhoA/Rock2-dependent phosphorylation of Vimentin likely represents one of the mechanisms through which ARHGAP19 controls cell shape changes in early mitosis of lymphocytes.
Discussion

We report here that ARHGAP19, a previously uncharacterized protein, is a critical actor of T lymphocyte division. The arhgap19 gene is expressed predominantly in cells of hematopoietic origin, with the noticeable exception of germ cells. This may explain why it has escaped identification in the previous, large scale siRNA-based screens seeking for genes involved in division of epithelial cells or fibroblasts. We observed that ARHGAP19 controls two aspects of lymphocyte division: time of morphological changes in early mitosis, and chromosome segregation in anaphase.

ARHGAP19 actions throughout cell division

The sole existing clues regarding the molecular actors controlling mitotic cell elongation in early mitosis come from studies using Drosophila S2 cells. In these cells, Rock or Myosin II depletion through siRNA impaired cell elongation in early anaphase, decreased mitotic cell blebbing and delayed cleavage furrow formation (Hickson et al., 2006). The morphological phenotypes we observed in lymphocytes overexpressing ARHGAP19 WT are reminiscent of those described when the Rock/Myosin II pathway is altered in S2 cells. In addition, we found that ARHGAP19 acts as a GAP for RhoA in early mitosis, and controls Citron and Myosin II recruitment at the plasma membrane, on which depends cytoskeleton contractility, as well as Rock2-mediated phosphorylation of Vimentin, a critical determinant in stiffness and shape of lymphocytes. This ARHGAP19-regulated RhoA/Rock2/Vimentin signaling pathway occurring in early mitosis of lymphocytes has not been described in adherent cells, in which pS71 Vimentin was found only during anaphase (in the ingressing furrow) or telophase (in the intercellular bridge) (Goto et al., 1998; Kosako et al., 1999; Yasui et al., 2001), and may be a characteristic of cells dividing in suspension.

In addition to cell elongation, cleavage furrow ingression also occurred earlier in ARHGAP19-deficient lymphocytes than in control cells. The excessively elongated shape in early mitosis could be sufficient to promote early furrow ingression by bringing into close proximity the spindle and the cell cortex (Rappaport and Rappaport, 1993; Shuster and Burgess, 1999; Shuster and Burgess, 2002). The increased recruitment of Myosin II at the plasma membrane, which favors cytoskeleton contractility, is an alternative or complementary explanation for early cleavage furrow ingression. In adherent cells, RhoA activity is required for de-adhesion and rounding up at mitosis onset. The obvious inference of our results is that, in lymphocytes where these steps are not needed, RhoA activity must be restrained to prevent
precocious morphological changes. We propose that some of the ARHGAP19-regulated pathways serve to delay these cell shape changes (including furrow ingression) until chromosomes segregate away from the cell midzone during anaphase.

In 43.6% ARHGAP19-deficient lymphocytes, lagging DNA was observed during furrow ingression. Others have shown that the CDC42 member of the Rho GTPases family, but not RhoA, controls chromosome segregation in other cell types, by regulating centromeres maintenance (Lagana et al., 2010), microtubules attachment to kinetochores (Oceguera-Yanez et al., 2005; Yasuda et al., 2004), as well as spindle morphology (Ban et al., 2004). The p190B RhoGAP-dependent modulation of Rac activity has also been shown recently to regulate attachment of microtubules to kinetochores (Hwang et al., 2012). However, ARHGAP19 did not act on CDC42 or Rac (Fig. 5A and B). Moreover, analysis of the spindle structure in ARHGAP19-deficient lymphocytes did not reveal major defects (Fig. S4 in supplementary material). Finally, staining of ARHGAP19-deficient lymphocytes with anti-phospho-CENPA or anti-BuBR1 antibodies revealed that both proteins are present on kinetochores during prophase, and absent in cells in anaphase, even in those with massive chromatid bridges (not shown). Thus, ARHGAP19 depletion does not seem to affect the activation of the spindle assembly checkpoint that normally occurs in early mitosis, or to allow mitotic slippage in the presence of an unsatisfied checkpoint (Brito and Rieder, 2006). Consistent with these notions, the lagging DNA events observed through videomicroscopy monitoring of ARHGAP19-depleted lymphocytes did not look like chromosomes “left behind” as a result of defective attachment of microtubules to centromeres or their detachment during chromosome poleward segregation. Instead, 44% of the ARHGAP19-deficient lymphocytes showed chromosome bridges occurring from the beginning of anaphase onset (in mild scenarios), or an apparent enlargement of the metaphasic chromosome plate as if sister chromatids were subject to forces pulling them apart but were not able to separate (in cells with the most severe phenotypes). These observations look similar to those described in cells with impaired Topoisomerase II activity (which results in inhibition of DNA decatenation and physically prevents sister chromatid separation)(Spence et al., 2007). How the RhoA hyperactivation that results from ARHGAP19 depletion may affect chromatin organization or chromosome separation remains to be elucidated.

Although the existence of variations in ARHGAP19 levels in leukemic T cells, revealed through our transcriptome analysis, might be circumstantial, we do not exclude that the genomic instability resulting from ARHGAP19 malfunction might participate in the leukemic
process. Further investigations will be required to characterize the mechanisms underlying the chromosome mis-segregation induced by ARHGAP19 silencing in lymphocytes.

**Spatiotemporal regulations of RhoA and ARHGAP19, and possible feedbacks**

Plasma membrane recruitment of RhoA was different in dividing lymphocytes expressing the WT or Rmut forms of GFP-ARHGAP19. This indicates that ARHGAP19 activity impacts on RhoA localization. In that plasma membrane recruitment of RhoA is known to reflect its activation status (Nishimura and Yonemura, 2006; Pickny et al., 2005), and that ARHGAP19 displays a GAP activity on RhoA, this observation was not surprising. Less expected was that the spatiotemporal plasma membrane recruitment of ARHGAP19 itself was linked to its activity and/or to downstream events. ARHGAP19 (endogenous, or fused to GFP in its WT or Rmut form) was nuclear before mitosis onset, scattered in the cytoplasm after nuclear envelop breakdown, and, at some point, re-localized to the plasma membrane (Fig. 2, 3C and 3E and Video 2 and 4). Strikingly, GFP-ARHGAP19 Rmut was recruited to the membrane earlier than GFP-ARHGAP19 WT, and whereas the main areas of GFP-ARHGAP19 Rmut recruitment were the cell equator and then the ingressing furrow, the most remarkable re-localization of GFP-ARHGAP19 WT was at the poles of the dividing lymphocytes.

During live observation of GFP-ARHGAP19 Rmut-expressing lymphocytes, plasma membrane recruitment of ARHGAP19 was always announcing imminent anaphase onset (in the cells dividing without major phenotypes) or precocious cell ingression. Analyses in fixed cells indicated that the zones of membrane recruitment of GFP-ARHGAP19 Rmut and RhoA globally coincided (Fig. S5 in supplementary material). Thus, the ability of active RhoA to interact with GAP proteins probably contributes to the recruitment of ARHGAP19 to the equatorial plasma membrane. Nevertheless, this model cannot account for the totality of our observations in GFP-ARHGAP19 WT-expressing dividing lymphocytes. The delay in membrane recruitment of GFP-ARHGAP19 WT could be explained by the decrease in RhoA activity. However, the re-localization of GFP-ARHGAP19 WT to the poles of the dividing lymphocytes, which was repeatedly observed both in live and fixed cells, was never accompanied by enrichment of RhoA in these areas. Thus, while RhoA activation levels likely influence membrane recruitment of ARHGAP19, additional mechanisms must be at play. Many of the Rho pathway proteins involved in mitosis are regulated by phosphorylation (David et al., 2012), suggesting that the dynamic re-localization of ARHGAP19 during the
course of mitosis may be under the control of one or several mitotic kinases. Regardless of the mechanisms responsible for GFP-ARHGAP19 WT sequestration to the cell poles, this phenomenon may represent a regulatory feedback through which depletion of ARHGAP19 from the furrow area would alleviate the interference with cleavage furrow ingression resulting from excessive GAP activity. Consistent with this hypothesis, re-localization of GFP-ARHGAP19 WT to the cell poles always preceded and was immediately followed by cleavage furrow ingression (Fig. 3C and Video 2). Restricted localization of endogenous ARHGAP19 to the poles was not observed in control cells (Fig. 2), possibly because such localization is transient in cells performing cytokinesis without phenotypic delays in morphological changes. Delineation of the mechanisms responsible for interaction of ARHGAP19 with the membrane and those that restrict ARHGAP19 to specific membrane areas will be required to determine whether ARHGAP19 recruitment to the cell poles occurs only in cells with excessive GAP activity, or also participates in focusing the zone of active RhoA in control cells (which is important for efficient furrowing).
Materials and Methods

Transcriptome analysis

The collection of 100 samples of human T-cell acute lymphoblastic leukemia and the microarray experiments were described previously (Soulier et al., 2005). We established a list of Affymetrix probes corresponding to 300 genes of the Rho GTPases pathways. Probes for which signals below background had been obtained in all the leukemia samples, as well as those that showed no significant variations across samples were deleted from the list. Unsupervised, hierarchical 2D clustering was then performed with this shortened list of probes.

Cell culture

Human Kit225 lymphocytes (Hori et al., 1987) were cultured as previously described (Seguin et al., 2009). Transfected cells were selected in 1-2 µg/ml puromycin. GFP-ARHGAP19 expression was induced by overnight treatment with 2 µg/ml doxycyclin. To block their cycle in the G1 phase, lymphocytes were cultured in the absence of IL-2 for 36-48 hours. Cell cycle progression was then resumed by addition of IL-2. To enrich the population in prometaphase-blocked cells, 40 ng/ml nocodazole (Sigma-Aldrich, St. Louis, MO, USA) was added for 16 hours (starting 24 hours after IL-2 addition). Cells were then washed thoroughly and placed in IL-2-containing culture medium to allow progression through mitosis.

Human leukemia Jurkat T cells (ACC 282) were obtained from DSMZ (Braunschweig, Germany) and cultured in Glutamax-containing RPMI 1640 medium supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. In experiments involving stable transfection, Jurkat cells were selected and maintained in 1 µg/ml puromycin.

HeLa cells were obtained from ATCC and cultured in Glutamax-containing DMEM medium supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. HeLa cells were transiently transfected using JetPEI transfection reagent (PolyPlus Transfection, Illkirch, France).

Plasmids

cDNA coding for human ARHGAP19 was generated by RT-PCR, using specific primers (5'-tcgaAGATCTATGGCGACTGAGGCAAGCAGG-3' and 5'-...
accgGAATTCTGCAAGGCACATAGGAGACA-3') and RNA from Kit225 lymphocytes as a template, and cloned into the pEGFP-C1 (Clontech, Mountain View, CA, USA) or pGEX4T2 (Pharmacia Biotech, Uppsala, Sweden) vectors between the BglII and EcoRI sites. Sequence analysis confirmed that the cloned ORF encodes the reference ARHGAP19 protein (SwissProt Q14CB8).

Where indicated, the R143 amino acid of ARHGAP19 was mutated using the QuikChange II XL Kit from Stratagene (La Jolla, CA, USA), the forward primer CAAAAACTTGCGAGTAGAGGGTTTGTTTgcAGTACCGGGTAATAG and its reverse complement.

The pEGFP-ARHGAP19ΔCter construct encoding the truncated form of ARHGAP19, in which the last 169 amino acids are missing, was generated by digesting the pEGFP-ARHGAP19 plasmid with BamHI and re-ligating it on itself.

In the pEGFP-Cter construct, the region encoding the C-terminal 169 amino acids of the canonical ARHGAP19 was placed in frame with the EGFP coding region of the pEGFP-C1 vector. For this purpose, the pEGFP-C1 vector was digested with BglII and BamHI, dephosphorylated using CIAP and gel-purified. In parallel, the pEGFP-ARHGAP19 construct was digested with BamHI and the resulting 522 nucleotides-long insert was gel-purified. Following ligation and plasmid amplification and purification, sequence analysis was performed to identify plasmids in which the 522 nucleotides-long insert was in the correct orientation.

To generate the plasmid allowing inducible expression of GFP-ARHGAP19, we digested the pEGFP-ARHGAP19 plasmid using the AgeI and EcoRI restriction enzymes and sub-cloned this insert into the pTRIPZ vector (OpenBiosystems, Thermo Fisher Scientific, Huntsville, AL, USA).

Silencing of ARHGAP19 expression was performed through transfection of pGIPZ plasmids from OpenBioSystems: the targeting sequences of the “Sh113” and “Sh117” short hairpins (mature antisenses) were TTAACCTTTGTGATATTCTC and TAGGTATTGAGGAATACAG, respectively.

**Purification of GST-ARHGAP19 protein and generation of anti-ARHGAP19 rabbit antibodies.**

KRX bacteria (Promega, Madison, WI USA) were transformed with the pGEX-ARHGAP19 plasmid. Expression of GST-ARHGAP19 was induced by IPTG for 4 hours at
Bacteria were lysed in a buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, 2 mM MgCl2, 0.5% Triton X100, 1 mM DTT, 2 mg/ml lysozyme and protease inhibitors, and sonicated. GST-ARHGAP19 fusion proteins were purified with glutathione-sepharose beads (GE Healthcare, Uppsala, Sweden) and then eluted in the presence of 25 mM reduced glutathione, 100 mM Tris pH 8, 150 mM NaCl, 1 mM DTT and 0.5% Triton X100. Dialysis of eluted proteins was performed in a buffer containing 100 mM sodium bicarbonate pH 8.4, 500 mM NaCl, 1 mM DTT and 0.5% Triton X100.

GST-ARHGAP19 proteins were used as immunogen for Eurogentec (Angers, France) to produce rabbit antisera. Antibodies were purified by incubating whole serum with GST-ARHGAP19 proteins covalently coupled to CNBR beads (GE Healthcare). After extensive wash of the beads with two distinct buffers (Buffer #1: 0.1 M Sodium Acetate pH 4, 0.5 M NaCl, 0.5% Triton X100; Buffer #2: 100 mM Sodium bicarbonate pH 8.4, 500 mM NaCl, 1 mM DTT and 0.5% Triton X100), antibodies were eluted with 20 mM glycine pH 2.5, and immediately supplemented with 0.1 volumes of 1 M Tris pH 8.5. Antibodies were then dialyzed in PBS overnight.

In vitro GAP assay

GFP-ARHGAP19 (WT or Rmut) was immunoprecipitated using GFP-Trap beads (Chromotek GMBH, Planegg-Martinsried, Germany) from doxycyclin-treated GFP-ARHGAP19-expressing Kit225 cells synchronized in prometaphase. Assay was performed using a kit from Cytoskeleton, Inc (Denver, CO, USA), according to the manufacturer protocol.

RT-PCR

Total RNA from Kit225 lymphocytes were purified and subjected to RT-PCR using primers specific for ARHGAP19, RacGAP1, ECT2, NET1, CCNA2 or GAPDH. Primer sequences were: GGCCATTGGTGAATTGAAGG and TGCATGGACCATAGGAGACA for ARHGAP19; CAGATAAGGGTCAATAGCAGTC and TCCCAACTAACAATAAAGAGTAGGC for RacGAP1; CATTTGCTGGTTTCAAAGTGTGA and GACAAACATTTTGTAGCACTCCC for Ect2; TAGTCATAAGATGAAAGAGACTG and GATCTATTTACAGTGCTTTTTC for Net1; AAATCTGTAAACATGAAAGACTG and GATAACCATAATTTGTACTTGGCCT for CCNA2; ACCACAGTCCATGCAACCTCAC and TCCACCACCTGTGCTGTA for
GAPDH. PCR products were run on agarose gels containing ethidium bromide and analyzed under UV using a GelDoc system (BioRad, Hercules, CA, USA).

**Antibodies**

Rabbit SY1985 anti-ARHGAP19 antibody was home-made. Goat anti-RACGAP1 (ab2270), rat anti-phospho-S71 Vimentin (TM71) and rabbit anti-phosho-Ser28-Histone H3 (ab32388) were from Abcam (Cambridge, UK). Rabbit anti-NET1 (ab2), rabbit anti-nonmuscle Myosin IIA and mouse anti-alpha-Tubulin (DM1A) were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-ECT2 (sc-1005), mouse anti-Hsc70 (sc-7298), goat anti-Citron (sc-1848), goat anti-ARHGAP19 (sc-104815) and mouse anti-RhoA (26C4) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-CDC42 (ACD03) was from Cytoskeleton Inc (Denver, CO, USA). Mouse anti-p190B RhoGAP (clone 54) and mouse anti-Rac1 (clone 102) were from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit anti-phospho-Ser1366-ROCK2 antibody (GTX122651) was from GeneTex (Irvine, CA, USA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare (Uppsala, Sweden) and Cell Signaling Technology (Ipswich, MA, USA), respectively. The secondary antibodies used for microscopy, conjugated to Alexa 488, Alexa 594 or Rhodamine Red, were from Molecular Probes (Eugene, OR, USA).

**Immunoprecipitations and Western blots**

Lymphocytes were lysed in 50 mM Tris pH 7.5, 0.5% NP40, 50 mM NaCl, 5 mM MgCl2, 10 mM NaF, 2 mM NaPP, 1 mM NaV, 1 mM DTT, 25 mM Calyculin A (#208851, Calbiochem, La Jolla, CA, USA), 25 mM Okadaic acid (#459618, Calbiochem), 10 µM Mg132 (#474790, Calbiochem), 1 mM PMSF and protease inhibitors (#P8240, Sigma-Aldrich). For analysis of WCL, 50 µg of proteins were used. Immunoprecipitation of endogenous ARHGAP19 (using 1 µg of purified SY1985 antibody, 1 mg of WCL and Protein G-Sepharose beads (GE Healthcare)), poly-acrylamide gel electrophoresis, wet transfer onto PVDF membranes (GE Healthcare) and Western blots were performed according to standard procedures.

**Video Microscopy**

Unsynchronized lymphocytes were treated with the cell permeable DNA stain syto59 (3.75 nM; Molecular Probes) and settled on a coverslip-like surface (Ibidi 60 µ-Dishes35mm, high; Ibidi, Martinsried, Germany) coated with a suboptimal dose of poly-L-lysine (0.002% in PBS; 18
Sigma-Aldrich) in order to limit their displacements during the time course of the experiment. Importantly, cells did not adopt an adherent morphology when using these settings. Cells were photographed every minute using the Fluoview FV10i-W confocal laser scanning microscope (objective 60 X W NA 1.2), equipped with a built-in incubator. The environment in the culture chamber was maintained at 37°C, 90% humidity and 5% CO2. Laser power was kept as low as possible (usually below 5%) to avoid potential detrimental effects on the cells. Acquired images were processed using the Image J software.

Microscopy on fixed cells
Lymphocytes were fixed (in suspension) for 15 min using a solution of 4% paraformaldehyde in PBS at 37°C, or for 10 min using a solution of 10% trichloroacetic acid in water at 4°C. Cells were washed in PBS, incubated in a solution of 0.05 M NH4Cl in PBS for 15 min and permeabilized with washing buffer (0.1% Triton X100, 3% BSA, in PBS) for 10 min. Staining was achieved by two consecutive, 1 hour incubations in solutions containing the primary and secondary antibodies diluted in washing buffer. Cells were re-suspended in 10 µl fluorescence-preserving mounting medium (Dako North America, Inc, Carpinteria, CA, USA) containing 2.5 µg/ml Hoechst 33342 (Molecular Probes) and placed between glass slides and 24 X 24 mm coverslips. Cells were examined under a Zeiss AxioImager Z1 fluorescence upright microscope (Zeiss, Jena, Germany) equipped with motorized Z drive, apotome and 63 X/1.40 oil M27 objective. Images were processed using the Image J software. For experiments involving HeLa cells, the same protocol was followed, except that cells were first seeded onto glass coverslips coated with fibronectin, before fixation. Where indicated, 3.3 nM Alexa Fluor®594-conjugated Phalloidin (Molecular Probes) was applied to the fixed cells for 1 hour, to stain polymerized actin-containing structures.

Statistical analyses
Data were processed using the GraphPad software to calculate mean ± s.e.m. Unpaired, two-tailed t tests were performed to determine whether means were significantly different.
Acknowledgments

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Author Contributions and Disclosure of Conflicts of Interest: M. D. D. and J. B. designed the study, analyzed the data and wrote the manuscript. M. D. D. carried out project planning and, together with D. P., performed the experiments. The authors declare no financial conflict of interest.
References


myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. Oncogene 19, 6059-64.


Figure Legends

Fig. 1. Expression of the arhgap19 gene co-varies with mitotic-related genes in human leukemia samples and fluctuates through cell cycle progression in human T lymphocytes. (A) Unsupervised, two-dimensional clustering of levels of expression of 300 genes of the Rho pathways (vertical) in 100 samples of human T-cell acute lymphoblastic leukemia (horizontal). In the heatmap, gene expression levels are color-coded, with red and blue corresponding to higher and lower levels, respectively. (B) shows the part of the dendrogram corresponding to the gene cluster “i” of the panel (A) and illustrates distances between genes. (C) List of the top-correlated genes (i.e. correlation coefficient above 0.74) of the global transcriptome that co-vary with the cluster “i” and, when stated in the GO database, mention of their involvement in mitosis and/or cytokinesis. (D, E) IL-2-dependent Kit225 cells were synchronized in G1 by factor deprivation, then stimulated to re-enter cell cycle by adding back IL-2. Where indicated, cells were blocked in prometaphase using nocodazole, washed, and cultured for up to 5.5 hours. RNA (D) or proteins (E) were purified from these cells and subjected to RT-PCR and Western Blots, respectively. (D) Levels of mRNA coding for ARHGAP19, RacGAP1, Ect2 and Net1 co-varied with that of Cyclin A2 (CCNA2), used here as an example of gene induced during the S-phase. GAPDH was used as a loading control. (E) Western blot analyses showed maximal expression of the ARHGAP19, RacGAP1, Ect2 and Net1 proteins in mitosis. Such cell-cycle-dependent regulation of expression is not a hallmark for all Rho pathway components, however, as level of the p190B RhoGAP protein remains constant during T lymphocyte cell-cycle progression. Hsc70 was used as a loading control.

Fig. 2. Localization of endogenous ARHGAP19 in interphase and throughout cell division. (A) Untransfected Kit225 lymphocytes were fixed with PFA and labeled for alpha-Tubulin (green), DNA (blue), and ARHGAP19 (red) using our home-made anti-ARHGAP19 antibody. Shown is a cell in interphase that stained positive for ARHGAP19. Of note, similar results were also obtained using the anti-ARHGAP19 from GeneTex, and the specificity of both these antibodies for ARHGAP19 was demonstrated by their inability to stain ARHGAP19-depleted lymphocytes (not shown). (B) Untransfected Kit225 lymphocytes were fixed with TCA and labeled for ARHGAP19 (red) and DNA (blue). In prophase (1) and metaphase (2), ARHGAP19 is dispersed in the cytoplasm. After anaphase onset, ARHGAP19
is recruited at the cell periphery (3). During cleavage furrow ingression and until the end of telophase (4 to 9), ARHGAP19 concentrates at the equatorial cortex.

**Fig. 3.** Manipulation of ARHGAP19 levels impacts on timing of cell elongation and cleavage furrow formation, as well as on mitotic blebbing and chromosome segregation in human lymphocytes. (A) Kit225 lymphocytes transfected or not (-) with the ShRNA 113 or 117 were synchronized in prometaphase using nocodazole. Efficiency of ARHGAP19 depletion was confirmed through immunoprecipitation followed by Western blot. Evaluation of Ect2 expression was used as an input control. (B-E) Mitosis progression of 18 control Kit225 lymphocytes (B), 25 GFP-ARHGAP19 WT-expressing Kit225 lymphocytes (C), 34 ARHGAP19-depleted Kit225 lymphocytes (D) and 35 Kit225 cells expressing GFP-ARHGAP19 Rmut (E) was followed by time-lapse microscopy. Selected frames of representative videos are displayed. The red color corresponds to fluorescence emitted by the DNA labeling, Syto 59 dye. In (C) and (E), the green color corresponds to the GFP signal. Time “0 min” was set on the last frame displaying the cell at the metaphase stage. (C) 100% of the GFP-ARHGAP19 WT-expressing cells showed significant delays in cell elongation and time of cleavage furrow formation as compared to control cells. (D) The upper panel illustrates the 56.4% of the ARHGAP19-depleted cells that performed a rather normal mitosis, although excessive blebbing can be noted. The middle and lower panels illustrate the defects observed in the remaining 43.6% of these cells, including precocious cell elongation and cleavage furrow ingression occurring before effective segregation of the chromosomes away from the furrow area. White arrows point out lagging DNA. (E) 34.3% of the GFP-ARHGAP19 Rmut cells performed rather normal division, although excessive blebbing can be noted (upper panel). The middle and lower panels illustrate the remaining 65.7% of these cells for which mitosis is severely perturbed, with phenotypic abnormalities reminiscent of those observed in ARHGAP19-depleted cells. This highlights the role of the GAP domain of ARHGAP19 in cell division control, and suggests that GFP-ARHGAP19 Rmut acts as a dominant negative. See also online Videos 1-4. (F, G) Quantitative analyses (mean ± s.e.m.) of the videos. (F) Cell length and cell width at the equator/furrow were measured every minute during mitosis progression. (G) Time of cell elongation or of cleavage furrow ingression were defined as the periods separating the last frame showing cells in metaphase and the first frame showing cells with an elongated morphology (length-width>2µm), or the first frame showing a furrow, respectively. Unpaired, two-tailed t tests were performed using
the GraphPad software to determine whether means significantly differed between cell populations. Of note, the ARHGAP19-deficient lymphocytes in which chromosome mis-segregation was so pronounced that the exact time of anaphase onset was difficult to determine were not included in these quantifications.

**Fig. 4. Manipulation of ARHGAP19 levels impact on cell shape, in cells blocked in prometaphase through treatment with nocodazole.** Lymphocytes (Control, ARHGAP19-overexpressing, or ARHGAP19-depleted) were synchronized in G1 by IL-2 withdrawal for two days, after which cell cycling was resumed by addition of IL-2 for 24 hours. Cells about to enter mitosis were then treated with nocodazole for five hours. Cells were fixed using PFA, and labeled for DNA (Hoechst, blue) and phospho-Histone H3 (red). (A) Representative fields for each population are shown. (B) For each population, more than 150 cells on random fields of three independent experiments were analyzed. The histogram shows mean ± s.e.m. percentages of elongated or round cells.

**Fig. 5. ARHGAP19 displays GAP activity on RhoA, but not Rac1 or CDC42.** (A) *In vitro* assay assessing the ability of ARHGAP19 to promote GTPase-mediated GTP hydrolysis. The histogram displays the amount of inorganic phosphate released by recombinant GTPases, in the absence or the presence of GFP-ARHGAP19 (WT or Rmut) purified by immunoprecipitation from mitotic Kit225 lymphocytes, or of recombinant p50RhoGAP used here as a positive control. (B-D) WT or Rmut forms of truncated GFP-ARHGAP19 (GFP-ARHGAP19-ΔCter) were expressed in HeLa cells. (B) Pulldown (PD) experiments using lysates of HeLa cells expressing the WT or Rmut forms of GFP-ARHGAP19-ΔCter. GST-Rho Binding Domain of Rhotekin (GST-RBD) or GST-CRIB of PAK (GST-CRIB) were used to pull-down active RhoA or active Rac1 and CDC42, respectively. Thus, Western blots on the PD lanes reveal levels of active GTPases, whereas Western blots on the WCL lanes show total levels of the indicated GTPases. As a positive control, untransfected HeLa cells were treated for 30 minutes with CNF1, a bacterial toxin that induces deamination of the catalytic glutamine of the Rho GTPases and thus prevents them from hydrolyzing GTP. (C, D) Transfected HeLa cells were seeded onto glass coverslips coated with laminin, and then fixed with PFA. (C) DNA and polymerized actin-containing structures were stained with Hoechst (blue) and Phalloidin (red), respectively. Expression of the WT, but not the Rmut form, decreased stress fibers occurrence, as compared to adjacent, untransfected cells. (D) At the
metaphase stage, untransfected HeLa cells (not shown) or cells expressing GFP-ARHGAP19-ΔCter Rmut (upper panel) were round and displayed a loosened adherence to the extracellular matrix, whereas cells expressing GFP-ARHGAP19-ΔCter WT (lower panel) remained fully adherent and flat. Histogram represents surface areas of these cells in metaphase (mean ± s.e.m; N=24 for each population).

**Fig. 6.** Manipulation of ARHGAP19 levels or activity affects the cell membrane recruitment of RhoA, Citron and Myosin II, especially during the first steps of mitosis (from prophase to early anaphase). Kit225 lymphocytes (control, expressing the WT or Rmut forms of ARHGAP19, or depleted in ARHGAP19) were fixed using TCA. Cells were labeled for DNA (Hoechst, blue) and RhoA (red, in A), Citron (red, in C) or Myosin II (green, in D). Each panel shows optical sections corresponding to the median plane of the mitotic cells. For the red and green channels, exposure times during picture acquisitions (linear mode) were kept constant from one population to the other. Moreover, image post-treatments (e.g. contrast adjustments) were performed after combination of all the individual pictures into one. Thus, relative intensities reflect actual variations of staining according to the mitotic stage or the status of ARHGAP19 expression or activity. (B) Quantification of RhoA recruitment to the membrane of dividing lymphocytes. For mitotic stages from metaphase to telophase, fluorescence intensities were measured at the equatorial membranes. For cells in prophase, fluorescence intensities were measured at random membrane positions. Average fluorescence intensities in the cytoplasm were subtracted from membrane fluorescence intensities to obtain values representing RhoA enrichment to membranes. Data are expressed as (mean ± s.e.m). N>10 for each mitotic stage of each cell population.

**Fig. 7.** Manipulation of ARHGAP19 levels or activity affects Rock2 activity as well as the phosphorylation status of its Vimentin substrate with which it colocalizes. Kit225 lymphocytes (control, expressing the WT or Rmut forms of ARHGAP19, or depleted in ARHGAP19) were fixed using TCA. Cells were labeled for DNA (Hoechst, blue), phospho-Rock2 (green) and phospho-S71 Vimentin (red). Shown in panel (A) is an overlay that reveals co-localization (yellow) of phospho-Rock2 and phospho-Vimentin. For the red and green channels, exposure times during picture acquisitions (linear mode) were kept constant from one population to the other. z-stack projections across the entire cell thickness are shown. Image post-treatments (e.g. contrast adjustments) were performed after combination of all the
individual pictures into one. Thus, relative intensities reflect actual variations of staining according to the mitotic stage or the status of ARHGAP19 expression or activity. Panel B is a higher magnification picture of the rectangle-delimited region of the ARHGAP19-depleted lymphocyte in prophase shown in A, which illustrates that intensity variations of the two signals do not always correlate (i.e. higher levels of red or green signals are detected depending on the portion of Vimentin filament considered).
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E) Release after Nocodazole block (hrs)

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- 200 kDa
- 75 kDa
A: Endogenous ARHGAP19, alphaTubulin, DNA

B: Endogenous ARHGAP19, DNA
Panel A shows images of cells under different conditions: Control, ARHGAP19 WT, and ARHGAP19 ShRNA. The images depict elongated and round cells.

Panel B presents a bar graph comparing the percentage of elongated and round cells across three conditions: Control, ARHGAP19 WT, and ARHGAP19 ShRNA. The graph indicates a significant increase in elongated cells and a decrease in round cells with ARHGAP19 ShRNA treatment compared to the Control and ARHGAP19 WT conditions.
A: In vitro GAP assay:

B: Protein expression analysis

C: HeLa cells in interphase:

D: HeLa cells in metaphase:

RhoA Rac CDC42

Journal of Cell Science Accepted manuscript
A: RhoA, DNA

B: Quantification of membrane recruitment of RhoA

C: Citron, DNA

D: Myosin IIA, DNA
A: 

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