Published in final edited form as:

Eur J Cell Biol. 2009 September ; 88(9): 495–507. doi:10.1016/j.ejcb.2009.04.005.

Constitutively active RhoA inhibits proliferation by retarding G₁ to S phase cell cycle progression and impairing cytokinesis

Pierre Morin^a, Cristina Flors^b, and Michael F. Olson^{a,*}

^aBeatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK

^bSchool of Chemistry, University of Edinburgh, Joseph Black Building, The King's Buildings, West Mains Road, Edinburgh EH9 3JJ, UK

Abstract

The actions of RhoA in cytoskeletal regulation have been extensively studied. RhoA also contributes to proliferation and oncogenic transformation by less well-characterized means. Elevated RhoA signalling has been associated with human cancer; through increased RhoA expression, mutation or elevated expression of activating Rho guanine-nucleotide exchange factors (GEFs), or from deletion or decreased expression of inhibitory Rho GTPase-activating proteins (GAPs). Unlike the Ras oncogene, constitutively-activated GTPase-deficient RhoA mutants have not been identified in tumours. To investigate the effects of active RhoA on proliferation, we generated Swiss3T3 cells that inducibly express wild-type RhoA or GTPase-deficient active V14RhoA. We found that V14RhoA inhibited cell proliferation by retarding entry into the DNA synthetic cell cycle phase and blocking successful completion of cytokinesis, resulting in an increased incidence of binucleate cells. These effects were associated with inhibition of mitogen-induced activation of the MAPK pathway, and suppression of several proteins involved in mitosis, including anillin, ECT2 and cyclin B1 which would be expected to result in reduced activation of endogenous RhoA at the cell equator. Accumulation of active RhoA protein in the midbody of cells in telophase was inhibited in V14RhoA-expressing cells, suggesting that RhoA inactivation must occur prior to re-activation. Defective cytokinesis was also associated with prominent actin structures in V14RhoA-expressing cells, which might be incompatible with equatorial furrowing. Using super-resolution imaging based on single-molecule switching, we have significantly improved the resolution of active RhoA in midbodies. These results indicate that constitutively-active RhoA antagonizes several cellular activities that contribute to proliferation, highlighting the importance for cycling between GTP/GDP-bound states.

Keywords

RhoA; Cell cycle; Actin; Cytoskeleton; Cytokinesis

© 2009 Elsevier GmbH. All rights reserved.

*Corresponding author: Tel.: +44 141 330 3654; m.olson@beatson.gla.ac.uk (M.F. Olson).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Rho family GTPases influence many biological processes including cell cycle progression and proliferation, with RhoA, Rac1 and Cdc42 being the most well-studied (Coleman et al., 2004). RhoGTPases cycle between inactive GDP-bound and active GTP-bound forms, with GTPase-activating proteins (GAPs) driving inactivation by accelerating GTP-hydrolysis, and guanine-nucleotide exchange factors (GEFs) promoting activation by facilitating GDP/GTP exchange (Jaffe and Hall, 2005). When GTP-bound, RhoA associates with effector proteins that relay signals and trigger biological responses. Through effector-protein recruitment, RhoA promotes increased polymerization and stabilization of filamentous (F)-actin, increased regulatory myosin light chain (MLC) phosphorylation and myosin ATPase activity, leading to contractile actin-myosin stress fibre formation (Leung et al., 1996; Uehata et al., 1997).

Although RhoGTPases have been associated with cancer (Sahai and Marshall, 2002; Gomez del Pulgar et al., 2005), activating Rho mutations have not been identified in tumours (e.g. (Fritz et al., 2002)). Instead, enhanced signalling may result from elevated RhoA levels, activating mutation or elevated expression of RhoGEFs, or from deletion or decreased expression of RhoGAPs (Kamai et al., 2002; Kleer et al., 2002; Sahai and Marshall, 2002; Gomez del Pulgar et al., 2005; Ellenbroek and Collard, 2007; Olson and Sahai, 2009). In contrast to the potent transforming-activity of RhoGEFs (e.g. (Miki et al., 1993)), mutant GTPase-deficient RhoA is a weak oncogene (e.g. (Avraham and Weinberg, 1989; Self et al., 1993; Khosravi-Far et al., 1995; Qiu et al., 1995; Lin et al., 1999; Sahai et al., 1999)). Although one interpretation is that RhoA needs to cycle between on- and off-states to produce dynamic signalling flux for transforming activity, an additional possibility is that the inability of GTPase-defective RhoA to be inactivated might have adverse effects that impair proliferation and oncogenic transformation.

Cell division is powered by a cortical actin-myosin contractile ring that forms equatorially and perpendicular to the mitotic spindle (reviewed in (Piekny et al., 2005)). The actin-myosin contractile ring results from localized RhoA activation. The RhoA effector anillin, which binds and coordinates F-actin and myosin (Piekny and Glotzer, 2008), is required for contractile ring function (Hickson and O'Farrell, 2008). Actin-myosin contractile force constricts the cell membrane to form the cleavage furrow (Piekny et al., 2005), continued contraction causes the cleavage furrow to ingress and the mitotic spindle to condense into a dense structure called the midbody, which keeps the two daughter cells connected via a thin intercellular bridge. Abscission results in the final intracellular bridge cleavage and daughter cell separation.

In order to determine how GTPase-deficient V14RhoA might affect proliferation, we generated Swiss3T3 mouse fibroblast cell lines expressing either wild-type or V14RhoA under the control of the tetracycline transcriptional activator (tTA). Induction of V14RhoA, but not wild-type RhoA, impaired proliferation associated with an increased incidence of binucleate cells, indicative of cytokinesis failure. In addition, V14RhoA expression retarded S-phase entry following re-stimulation of quiescent cells. These results indicate that GTPase-deficient V14RhoA has negative properties detrimental for oncogenic transformation, and that successful cytokinesis is dependent upon RhoA cycling between GDP/GTP-bound states.

Materials and methods

Plasmids

V14RhoA (containing an N-terminal myc-epitope) was PCR-amplified from pEF V14RhoA (Hill et al., 1995) using primers RHOA-6S and RHOA-7A detailed in Table 1. The PCR product was subcloned into BamHI and HindIII sites in pRevTRE (BD Biosciences). WTRhoA was PCR-amplified from V14RhoA in 2 steps. First, 2 PCR products corresponding to the N- and

C-terminal parts were generated using RHOA-6S + MutRHOA-2A and RHOA-7A + MutRHO-1S primer pairs detailed in Table 1. A *NarI* site (underlined) reintroduces wild-type Gly14. These 2 products were ligated together at the *NarI* site and subcloned into pRevTRE. All constructs were verified by sequencing.

Generation of V14RhoA and wild-type RhoA cell lines

Swiss3T3 cell lines were maintained in DMEM medium containing 10% donor calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). pRevTet-Off-IN (BD Biosciences), pRevTRE-V14RhoA and pRevTRE-WTRhoA were transfected into BOSC23 retroviral packaging cells (Pear et al., 1993) with Effectene (QIAGEN) according to manufacturer's instructions. After 36 h, supernatants were collected and centrifuged at 1600 rpm for 15 min, and stored at -80°C. Exponentially growing Swiss3T3 cells were transduced with pRevTet-Off-IN retrovirus and selected with 800 µg/ml G418 (Sigma). A stable founder line was selected for tTA highest expression and transduced with pRevTRE-V14RhoA or pRevTRE-WTRhoA retrovirus. After selection with 200 µg/ml hygromycin B (Sigma), clones were chosen for high and homogeneous WTRhoA or V14RhoA expression in the absence of tetracycline, with minimal protein levels in the presence of tetracycline).

Western blotting

Cells (0.5×10^6) were seeded in 10 cm diameter dishes and serum-starved for 24 h in the absence or in the presence of 1 µg/ml tetracycline. Cells were subsequently stimulated with 10% serum with or without 1 µg/ml tetracycline for indicated times. Cells lysates were Western blotted as detailed previously (Coleman et al., 2001) with: anti-myc tag (2276), anti-MEK1/2 (9122), anti-phospho-MEK1/2 (Ser217/221) (9121), anti-phospho-ERK1/2 (Thr202/Tyr24) (9106), anti-cyclin B1 (4135) and anti-myc (9402) from Cell Signaling; anti-cyclin D1 (sc450), anti-cyclin A (sc596), anti-anillin (sc67327), anti-ECT2 (sc1005) and anti-Raf1 (sc133) from Santa Cruz; anti-phospho-Raf1 (Ser338) from Upstate Biotechnology; anti-ERK2 from C.J. Marshall, Institute of Cancer Research, London UK. Membranes were probed with appropriate Alexa Fluor-conjugated secondary antibodies for 1 h, and immunoreactivity was visualized with a LI-COR Odyssey.

Real-time PCR

V14RhoA-Swiss3T3 cells (0.5×10^6) in 10 cm dishes were serum-starved for 24 h with or without 1 µg/ml tetracycline, then stimulated with 10% serum for indicated times with or without tetracycline prior to trypsinization. Cells were pelleted by centrifugation at 1000g for 15 min. Total RNA was prepared using the Qiagen RNeasy Kit according to manufacturer's instructions. RNA (5 µg) was reverse-transcribed using Superscript (Invitrogen) and oligo-dT. cDNAs were amplified by real-time (RT)-PCR using iTaq SYBR Green Supermix (Bio-Rad) and the following primers detailed in Table 1: MuGAPDH-1S; MuGAPDH-2A; RhoA-1S; RhoA-2A; MucycD-1S; MucycD-2A. RT-PCR was performed with the Chromo4™ RT-PCR Detector (Bio-Rad). Each condition was done in triplicate and the experiment repeated with 5 independent mRNA preparations. The relative expression differences between conditions expressed as mean $\pm$ standard error.

Cell cycle analysis by FACS

V14RhoA-Swiss3T3 cells were serum-starved for 24 h with or without tetracycline, and with or without Y-27632. Cells were stimulated with 10% serum with or without tetracycline, and with or without Y-27632, for 16 or 24 h. Cells were fixed with ice-cold 70% ethanol for 20 min, then treated with 3N HCl containing 0.5% Triton X-100 for 20 min. Cells were washed four times with PBS, incubated with propidium iodide (5 mg/ml) for 30 min, then analyzed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

Cell growth assays

Cells were seeded at 5–10% confluency in 24-well plates (175 mm²) with tetracycline concentrations ranging from 0 to 1000 ng/ml for 24, 48 or 72 h. Medium was changed daily to maintain constant tetracycline concentrations. Cells were trypsinized and counted using a CASY cell counter (Innovatis). Each condition was performed in triplicate and expressed as mean $\pm$ standard error.

Immunofluorescence studies

Cells were fixed and stained as detailed previously (Coleman et al., 2001) with: anti-RhoA (sc179; Santa Cruz), anti- α -tubulin (T5168; Sigma), anti-phosphoSer19-MLC (3675) and anti-myc tag (2276) from Cell Signaling. After 1 h, cells were washed twice with PBS before incubation with FITC-, Cy5- or Texas-red-conjugated secondary antibody diluted in 3% BSA/PBS for 1 h. F-actin was stained with Oregon green phalloidin (O-7465; Molecular Probes). Cells were washed twice with PBS and once with H₂O before mounting in Vectashield medium containing DAPI (Vector Laboratories) under glass coverslips. Cells were visualized with a Zeiss Axioplan2 microscope. For RhoA immunostaining, cells were fixed on ice with ice-cold 10% TCA for 15 min as previously described (Yonemura et al., 2004; Yuce et al., 2005; Nishimura and Yonemura, 2006).

Time-lapse microscopy experiments

Cells were seeded into 6-well glass bottom plates (Iwaki) at 5–10% confluency with or without 1 μ g/ml tetracycline. Pictures were taken every 10 min for 48 h using a motorized Zeiss time-lapse microscope equipped with a temperature- and CO₂-regulated environmental chamber. Images were processed using Andor IQ software.

Super-resolution fluorescence microscopy

The super-resolution imaging scheme used in this study was similar to direct stochastic optical reconstruction microscopy (dSTORM) (Heilemann et al., 2008), i.e. taking advantage of the blinking of cyanine dyes in a “switching buffer” (see below). However, no photoactivation laser was used, so we relied on the spontaneous activation of the fluorescence (Folling et al., 2008). Imaging was performed on a Nikon Eclipse TE2000 inverted microscope, equipped with a total internal reflection fluorescence (TIRF) oil-immersion objective (Apochromat, 60 $\times$, NA 1.49, Nikon). Excitation was provided by a 633-nm He/Ne CW laser (Coherent model #31-2140-000, 22 mW at the sample) passing through a z633/10 $\times$ bandpass filter (Chroma Technology). Wide-field oblique illumination was achieved by focusing the expanded and collimated laser beam onto the back-focal plane of the objective. Emission was collected by the same objective and imaged by an Andor Luca (S) EMCCD camera after passing through a dichroic mirror (z633rdc, Chroma Technology) and additional spectral filters (HQ645LP, HQ700/75, Chroma Technology). Additional lenses resulted in a final magnification of 198 $\times$, equivalent to a pixel size of about 74 nm. Integration time per frame was 100 ms, and the total number of frames collected was 3000. The images were analyzed with a MATLAB routine by fitting Gaussian functions to individual molecules and finding their centre, as described previously (Muls et al., 2005). The α -tubulin-FITC standard wide-field image was generated in the above setup by excitation with a 488-nm CW Ar⁺ laser (163-C, Spectra-Physics, 3 mW at the sample) passing through the appropriate filters, with 100 ms integration time.

Cells were imaged in a CoverWell imaging chamber (Grace Bio Labs), which contained “switching buffer”: PBS with an oxygen scavenger (0.5 mg/ml glucose oxidase (Sigma), 40 μ g/ml catalase (Sigma) and 10% w/v glucose (Fischer Scientific) and 50 mM β -mercaptoethylamine (MEA, Fluka) (Heilemann et al., 2008).

Results

Establishment of tetracycline-inducible V14RhoA-expressing cells

We generated Swiss3T3 mouse fibroblasts expressing GTPase-deficient constitutively-active RhoA (V14RhoA) or wild-type RhoA (WTRhoA), controlled by a tetracycline-regulated promoter (Tet-Off). In tetracycline-free medium, tetracycline transcriptional activator (tTA) promotes increased RhoA expression via tetracycline-responsive elements (TRE) upstream of a minimal CMV promoter. No signal was detected in the presence of tetracycline by immunofluorescence analysis for myc-epitope tagged V14RhoA, indicating stringent transcriptional repression (Fig. 1a, left panel). Following tetracycline withdrawal, V14RhoA was homogeneously induced (Fig. 1a, right panel). Similar results were obtained for WTRhoA (data not shown). It has been well established that ROCK kinases, downstream effectors of RhoA, play key roles in actin stress fibre formation by phosphorylating MLC and by stabilizing F-actin (Riento and Ridley, 2003). In order to assess the effect of V14RhoA induction on the actin-myosin cytoskeleton, immunofluorescence studies were performed on fixed cells with phalloidin to stain F-actin and anti-phospho-MLC antibody. As shown in Fig. 1b, V14RhoA induction following tetracycline withdrawal efficiently promoted actin stress fibre formation and MLC phosphorylation. V14RhoA also increased cell-cell adhesion contacts (compare top and middle panels). Furthermore, all these events were mediated in a ROCK-dependent manner, as demonstrated by their sensitivity to the ROCK selective inhibitor Y-27632.

V14RhoA induction reduces proliferation, slows S phase entry and blocks cells in G₂/M

We next analyzed the consequence of V14RhoA induction on proliferation. Cells were incubated in the absence or presence of tetracycline, with or without ROCK inhibitor Y-27632, and cell number was determined 24, 48 or 72 h later (Fig. 2a). V14RhoA strongly inhibited cell proliferation in a Y-27632-insensitive manner. It should be noted that ROCK inhibitor itself slightly reduced cell proliferation in control cells. To determine whether the anti-proliferative effect was specific for the GTPase-deficient V14RhoA, we compared the relative effects of WTRhoA and V14RhoA induction on cell number using concentrations of tetracycline ranging from 0 to 1000 ng/ml (Fig. 2b). After 24 or 48 h, WTRhoA and V14RhoA expression levels were monitored by Western blotting with anti-myc epitope antibody and their effects on cell proliferation were analyzed (Fig. 2c). WTRhoA did not affect cell proliferation following complete tetracycline withdrawal, whereas V14RhoA inhibited cell proliferation in a dose-dependent manner, even at tetracycline concentrations that induced V14RhoA expression to lower levels than WTRhoA (e.g. 5- or 18-fold induction of V14RhoA at 7 and 3.5 ng/ml tetracycline, respectively, versus 27-fold induction of WTRhoA in the absence of tetracycline at 48 h). These results indicate that the constitutively-active properties of the V14RhoA GTPase-deficient mutant were responsible for the anti-proliferative effects observed.

In order to determine where in the cell cycle V14RhoA might exert its anti-proliferative effects, cells were first synchronized in G₀/G₁ by serum-starvation for 24 h in the absence or in the presence of tetracycline, then stimulated with medium containing 10% foetal calf serum for 16 or 24 hours, followed by fixation, staining with propidium iodide and FACS analysis for DNA content (Fig. 3). Mean data from 5 independent experiments revealed that V14RhoA induction following tetracycline withdrawal did not affect cell synchronization in G₀/G₁ (i.e. 2N DNA content) following serum-starvation. However, 16 h after serum stimulation, only $57.8 \pm 6.9\%$ of V14RhoA-expressing cells had progressed into S phase compared to $70.1 \pm 11\%$ of control cells, indicating that V14RhoA induction slowed S phase entry (Fig. 3b). The proportion of cells remaining in G₀/G₁ following V14RhoA induction ($27.8 \pm 6.8\%$) was significantly greater ($p < 0.05$) than without V14RhoA induction ($17.2 \pm 6.2\%$). FACS analysis also revealed that V14RhoA induction significantly increased the percentage of cells in G₂/M

(4N DNA content) after 24 h of serum-stimulation ($30.9 \pm 8.3\%$ without tetracycline versus $15.2 \pm 2.1\%$ with tetracycline, $p < 0.01$). These results, together with the effects on cell proliferation (Fig. 2) suggest that the anti-proliferative effects of V14RhoA induction result from the combination of delayed S phase entry and G₂/M blockade. Consistent with the inability of ROCK inhibition to antagonize the negative effects of V14RhoA on proliferation, neither delayed S phase entry nor G₂/M blockade could be rescued by Y-27632 (data not shown).

V14RhoA induction reduces MAPK activation

We next investigated the possibility that V14RhoA affected cell cycle progression by modulating the Raf-MEK-ERK mitogen-activated protein kinase (MAPK) cascade using anti phospho-ERK1/2 (Thr185/Tyr187), anti phospho-MEK1/2 (Ser217/Ser221) and anti phospho-Raf1 (Ser338) antibodies which reflect the activation state of each protein (Fig. 4a). Chronic MAPK activation in response to serum-stimulation was transient, peaking at 3 h (Fig. 4a). Interestingly, in the presence of elevated V14RhoA, serum-induced phosphorylation of ERK, MEK and Raf1 were significantly reduced (Fig. 4b, $p < 0.01$). ERK proteins were only phosphorylated to $54 \pm 19\%$ and $51 \pm 15\%$, at 1 and 3 h, respectively, relative to control cells without V14RhoA induction (Fig. 4b). At the same time, MEK proteins were only phosphorylated to $63 \pm 30\%$ and $62 \pm 32\%$ and Raf1 to $81 \pm 12\%$ and $87 \pm 11\%$ at these time points following V14RhoA induction. When the effects of V14RhoA induction were examined at earlier time points, acute ERK and MEK activation at 5 and 15 minutes were not significantly affected (Fig. 4c). However, the second sustained phase of ERK activation was reduced between 30 minutes and 3 hours in cells expressing V14RhoA (Fig. 4d, $p < 0.01$). These results indicate that V14RhoA inhibits the sustained phase of serum-induced MAPK pathway activation, which is required for S phase entry (Yamamoto et al., 2006). Previous research has shown that the sustained phase of ERK activation is required for induction of cyclin D1, a key cell cycle regulator (Weber et al., 1997; Cook et al., 1999). When mRNA levels were examined by RT-PCR, tetracycline withdrawal was found to produce a ~2.5-fold induction in V14RhoA expression relative to GAPDH expression at all time points (Fig. 4e, upper panel). Cyclin D1 levels rose steadily in response to serum-stimulation in control cells in the presence of tetracycline (Fig. 4e, lower panel). However, V14RhoA induction following tetracycline withdrawal was accompanied by transient induction of cyclin D1, with greatly lower levels at later time points. These data indicate that the reduction in the sustained phase of ERK activation following V14RhoA induction resulted in lower expression of a key cell cycle regulatory protein, which likely contributes to the delayed S phase entry.

V14RhoA induces cytokinesis defects associated with the formation of binucleate cells

FACS analysis of cell cycle progression indicated an accumulation of cells in G₂/M phases in V14RhoA-expressing cells (Fig. 3), which was insensitive to the ROCK inhibitor Y-27632 (data not shown). Since it did not affect the G₂/M blockade, Y-27632 was used to promote cell spreading to make possible accurate nuclei counting. The apparent G₂/M blockade was correlated with an increased incidence of binucleate cells (Fig. 5a), comprising approximately 30% of the entire cell population 48 h after tetracycline withdrawal. In order to determine what stages of cell division were affected, we carried out time-lapse microscopy on cells that did or did not express elevated V14RhoA. Cells incubated with or without tetracycline were imaged every 10 min for 48 h. Without V14RhoA induction (plus tetracycline), cells completed division in approximately 1 to 1.5 h (Fig. 5b, upper panels). Cells rounded up, cleavage furrows formed at the cell equator and ingressed, and finally daughter cells underwent abscission. In contrast, following V14RhoA induction by tetracycline withdrawal, two types of cytokinesis defects were observed (Fig. 5b, middle and lower panels, respectively): an early one characterized by a failure of cleavage furrow formation, and a late one characterized by a failure of the abscission process (note the higher light intensity between cells due to the lower

refractive index of the gap relative to the higher refractive index of the cytoplasm). After monitoring numerous cell division events by time-lapse microscopy, we found that V14RhoA mainly induced the early failure of cleavage furrow formation (~90% of the events). However, it should be noted that the late cytokinesis defect might be underscored, since cells that appeared to successfully complete cell division might actually still be linked by an intracellular bridge not apparent by phase-contrast microscopy.

V14RhoA represses cytokinesis-regulatory proteins and fails to accumulate to the midbody in telophase

In order to investigate how V14RhoA affected cytokinesis, we Western blotted for several proteins that play critical roles in cytokinesis and whose expression fluctuate during progression towards G₂/M cell cycle phases (Zhao and Fang, 2005a; Saito et al., 2003), ECT2, a RhoA-specific GEF previously shown to be critical for RhoA activation during cytokinesis (Tatsumoto et al., 1999; Kim et al., 2005; Zhao and Fang, 2005b; Chalamalasetty et al., 2006); anillin, a RhoA effector protein that binds to and coordinates filamentous actin and myosin in the contractile ring (Piekny and Glotzer, 2008); and cyclin B1, an activator of Cdk1 that is required for mitosis entry and which phosphorylates ECT2 leading to its recruitment to the mitotic central spindle (Yuce et al., 2005). Cells were serum-starved for 24 h in the absence or presence of tetracycline, and then serum-stimulated for 16 to 23 h without or with tetracycline (Fig. 6a). In the absence of V14RhoA induction, serum-induced expression of ECT2, anillin and cyclin B1 remained relatively constant between 16 and 19 h and then slowly decreased, returning to basal expression levels at 23 h. In contrast, induction of ECT2, anillin and cyclin B1 were repressed in V14RhoA-expressing cells. Thus, V14RhoA expression inhibited the induction of several proteins with key roles in mitosis, which likely contributed to the failure of these cells to complete cytokinesis.

Since the RhoGEF ECT2 expression was strongly repressed by V14RhoA expression, we examined the cellular localization of active RhoA during mitosis by immunofluorescence analysis using a trichloroacetic acid fixation method that selectively fixes membrane bound (and therefore active) RhoA as previously described (Yonemura et al., 2004; Yuce et al., 2005; Nishimura and Yonemura, 2006; Dai et al., 2007). In control cells, RhoA was activated during mitosis and was diffusely localized in metaphase (Fig. 6b, upper left panel). Active RhoA next accumulated at the cell equator in anaphase and ultimately was restricted to a very small region in the midbody in late telophase (Fig. 6b, upper middle and right panels). In contrast, following V14RhoA induction active RhoA was detected throughout the cell, displaying no sign of accumulation to the cell equator or to the midbody (Fig. 6b, second row). Furthermore, we noticed that midbodies were frequently off-centre, suggesting that during cleavage furrow ingression there might have been disequilibrium of contractile forces due to unequal distribution of active RhoA. In comparison, active RhoA accumulation to the cleavage furrow and midbody occurred normally in WTRhoA-induced cells (Fig. 6b, lower panels). In addition, midbodies were properly centred in cells expressing elevated WTRhoA. We used super-resolution imaging (SRI) based on single-molecule switching in order to improve the resolution of active RhoA in midbodies. As shown in Figure 6c, conventional fluorescence microscopy can only resolve RhoA to a bright band in the midbody, however SRI revealed that the bright band is actually comprised of numerous discrete punctae. These results suggest that an excess of active RhoA may negatively feedback to inhibit expression of proteins such as ECT2 and anillin, and repress the localized activation of RhoA that is required for cytokinesis.

Discussion

Mutations that make Ras proteins GTPase-deficient are among the most frequent in human tumours. Analogous mutations facilitated characterization of RhoA function in the regulation of the actin cytoskeleton (Garrett et al., 1989; Paterson et al., 1990; Ridley and Hall, 1992). In contrast to the potency of active Ras in fibroblast transformation assays, constitutively-active RhoA has weak transforming ability (Avraham and Weinberg, 1989; Self et al., 1993; Khosravi-Far et al., 1995; Qiu et al., 1995; Lin et al., 1999; Sahai et al., 1999). However, activated RhoGEFs, which promote GTP loading onto RhoA, potentially transform mouse fibroblasts (Olson, 1996; Rossman et al., 2005) as does mutant RhoA that rapidly cycles between GDP/GTP-bound states (Lin et al., 1999). The absence of activating RhoA mutations in cancer and the failure of GTPase-deficient RhoA to mimic the effects of elevated active RhoA-GTP on proliferation and transformation suggest that the inability to cycle between GDP/GTP-bound states might impair proliferation and/or could be selected against during oncogenesis.

To investigate whether GTPase-deficient RhoA had negative effects on proliferation, we generated cell lines inducibly-expressing wild-type or V14RhoA. We observed a dose-dependent inhibition of proliferation in V14RhoA-expressing cells, but not in wild-type RhoA-expressing cells even at the highest induction levels. V14RhoA had a small but significant effect on delaying progression from G₀/G₁ into S phase, and significant accumulation in the G₂/M phase accompanied by a dramatic increase in binucleate cells indicative of cytokinesis failure. These results indicate that V14RhoA does have properties that inhibit proliferation, which would be incompatible with oncogenic transformation.

When V14RhoA was induced prior to serum-stimulation, we observed attenuation of the sustained phase of MAPK activation accompanied by delayed S phase entry. None of these effects was altered by the ROCK inhibitor Y-27632. These results were similar to the observation that microinjection of V14RhoA into serum-stimulated NIH 3T3 cells modestly reduced S phase entry (Molnar et al., 1997). The greatest negative effect of V14RhoA on the MAPK pathway was observed at the level of ERK phosphorylation, with lesser effects observed for MEK and Raf1 phosphorylation (Fig. 4b). One likely explanation is that due to the signal amplification that occurs down the kinase cascade, the modest inhibition in Raf1 phosphorylation is amplified at each step resulting in progressively greater decreases in MEK and subsequently ERK phosphorylation. The initial activation of MAPK was not affected by V14RhoA, the inhibition observed occurred during the second sustained phase, which has been shown to be important for entry into S phase (Cook et al., 1999). The second sustained phase has been shown to be critical for cyclin D1 induction (Villanueva et al., 2007), and consistent with V14RhoA repression of sustained ERK activity, cyclin D1 mRNA levels were lower in V14RhoA-expressing cells following serum-stimulation (Fig. 4e). These results suggest that the delayed entry into S phase was the result of suppressed activation of sustained ERK activity and consequent reduction in cyclin D1 induction.

The most significant effect of V14RhoA induction was an accumulation of cells in G₂/M and increased incidence of binucleate cells indicative of impaired cytokinesis (Fig. 3 and Fig 5), consistent with the previous finding of cytokinesis defects following injection of V14RhoA into *Xenopus* embryos (Drechsel et al., 1997). Time-lapse microscopy revealed two types of cytokinesis defects: an early one observed in ~90% of the cases that was characterized by a rapid failure of cleavage furrow formation or ingression, and a late one characterized by a failure of abscission and cell separation (Fig. 5). In this latter case, following the failure to divide the two daughter cells fused back into a single binucleate cell. These results suggest that V14RhoA inhibits cytokinesis at two stages.

The predominant stage at which V14RhoA affected cytokinesis was early, during cleavage furrow formation and/or ingression. It has been well documented that during cytokinesis active RhoA concentrates at the equatorial cell cortex (Takaishi et al., 1995; Yonemura et al., 2004; Nishimura and Yonemura, 2006). The current model is that equatorial localization of the ECT2 RhoGEF regulates this subcellular distribution of active RhoA (Chalamalasetty et al., 2006; Nishimura and Yonemura, 2006; Yuce et al., 2005). Knockdown of ECT2 by RNA interference or neutralizing antibody microinjection has been shown to be sufficient to induce cytokinesis arrest (Tatsumoto et al., 1999; Kim et al., 2005; Zhao and Fang, 2005b; Chalamalasetty et al., 2006). This model also pre-supposes that RhoA activity is effectively suppressed in the remaining regions of the cell to sufficiently low levels that allow for a degree of cytoskeletal plasticity, so that the contractile forces generated at the cell equator exceed cytoskeletal stiffness resulting in furrow formation and ingression. If V14RhoA activity were too great throughout the cell then the actin-myosin contractile ring might not be sufficiently strong to re-shape the cytoskeleton. Consistent with this interpretation, active V14RhoA was reproducibly observed throughout the cell during anaphase without noticeably more at the cell equator (Fig. 6b). Although induction of wild-type RhoA resulted in increased active RhoA distributed at the cell periphery, it was still possible to detect a higher concentration of active RhoA at the cleavage furrow and midbody. In *Xenopus* embryos, V14RhoA impaired cleavage furrow ingression and it was postulated that the furrowing defect might be due to active RhoA inducing the formation of a thick and stiffened F-actin rich cortex that resisted deformation by the ingressing furrow (Drechsel et al., 1997). In addition, the reduced induction of anillin protein in V14RhoA-expressing cells might contribute to the frequent failure of cleavage furrow formation and/or ingression (Fig. 6a). In anillin knockdown cells, cleavage furrows were initiated but then regressed, and it has been suggested that anillin promotes furrow stabilization by linking the contractile ring with the spindle microtubules (Straight et al., 2005; Zhao and Fang, 2005a; D'Avino et al., 2008; Gregory et al., 2008). An additional possibility is that the reduced induction of anillin in V14RhoA-expressing cells affects both ingression and abscission. In a search for factors involved in the maintenance and resolution of the intracellular bridge using an RNAi screen in *Drosophila* S2 cells, anillin was identified as essential for the terminal post-furrowing events of cytokinesis (Echard et al., 2004).

When ingression of the cleavage furrow is complete, active RhoA is restricted to a very small region in the middle of the midbody (e.g. Fig. 6b top panels). We show here that V14RhoA fails to localize to the midbody following ingression (Fig. 6b). In contrast, induction of WTRhoA did not result in cytokinesis defects, nor did it affect localization of RhoA to the midbody even when exhibiting an excess of RhoA at the cell periphery during mitosis (Fig. 6b, lower panels). These data suggest that it may be necessary for RhoA to be inactivated, possibly by the RhoGAP CYK-4/MgcRacGAP, before it can be re-activated at the midbody by ECT2. The inability of V14RhoA to localize to the midbody might, therefore, result from its resistance to GAP-mediated deactivation (Garrett et al., 1989). The requirement for RhoA deactivation for subsequent localized activation during cytokinesis is similar to the lack of RhoA activation at the equatorial cell cortex in mitotic cells depleted of CYK-4/MgcRacGAP by siRNA-mediated knockdown (Yuce et al., 2005). An additional contributing factor in V14RhoA-expressing cells is that the reduced induction of ECT2 and cyclin B1, which normally would associate with CDK1 to promote phosphorylation and activation of ECT2 (Hara et al., 2006) and GEF-H1 (Birkenfeld et al., 2007), results in failure to activate endogenous RhoA at the midbody. The attenuated RhoA activation resulting from reduced ECT2 protein and activity might also contribute to the low frequency of cleavage furrow formation in V14RhoA-expressing cells (Fig. 5b).

The results of this study suggest that expression of constitutively-active RhoA is incompatible with cell proliferation, mainly because of impaired cytokinesis. These results may also explain why constitutively activated mutants of RhoA have not yet been described in cancer. It appears

that high levels of active RhoA are not incompatible with proliferation and transformation, given that activated RhoGEFs and fast-cycling RhoA mutants are potent transforming oncogenes in experimental systems, and human cancers have been associated with activation of the LARG RhoGEF or loss of the DLC1 RhoGAP. These data support the conclusion that the ability to be inactivated, as well as activated, is critically important for RhoA function in cell proliferation and transformation.

Acknowledgements

This work was supported by Cancer Research UK (MFO), by a grant CA-030721 from the National Cancer Institute (MFO) and by the EPSRC Life Sciences Interface program EP/F042248/1 (CF). We thank Dr. H. Uji-i (KU Leuven, Belgium) for the software to reconstruct the super-resolution images.

References

- Avraham H, Weinberg RA. Characterization and expression of the human rhoH12 gene product. *Mol. Cell. Biol* 1989;9:2058–2066. [PubMed: 2501657]
- Birkenfeld J, Nalbant P, Bohl BP, Pertz O, Hahn KM, Bokoch GM. GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases. *Dev. Cell* 2007;12:699–712. [PubMed: 17488622]
- Chalamalasetty RB, Hummer S, Nigg EA, Sillje HH. Influence of human Ect2 depletion and overexpression on cleavage furrow formation and abscission. *J. Cell Sci* 2006;119:3008–3019. [PubMed: 16803869]
- Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat. Cell Biol* 2001;3:339–345.
- Coleman ML, Marshall CJ, Olson MF. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nat. Rev. Mol. Cell Biol* 2004;5:355–366. [PubMed: 15122349]
- Cook SJ, Aziz N, McMahon M. The repertoire of fos and jun proteins expressed during the G1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. *Mol. Cell. Biol* 1999;19:330–341. [PubMed: 9858557]
- D'Avino PP, Takeda T, Capalbo L, Zhang W, Lilley KS, Laue ED, Glover DM. Interaction between Anillin and RacGAP50C connects the actomyosin contractile ring with spindle microtubules at the cell division site. *J. Cell Sci* 2008;121:1151–1158. [PubMed: 18349071]
- Dai BN, Yang Y, Chau Z, Jhanwar-Uniyal M. Polo-like kinase 1 regulates RhoA during cytokinesis exit in human cells. *Cell Prolif* 2007;40:550–557. [PubMed: 17635521]
- Drechsel DN, Hyman AA, Hall A, Glotzer M. A requirement for Rho and Cdc42 during cytokinesis in *Xenopus* embryos. *Curr. Biol* 1997;7:12–23. [PubMed: 8999996]
- Echard A, Hickson GR, Foley E, O'Farrell PH. Terminal cytokinesis events uncovered after an RNAi screen. *Curr. Biol* 2004;14:1685–1693. [PubMed: 15380073]
- Ellenbroek SI, Collard JG. Rho GTPases: functions and association with cancer. *Clin. Exp. Metastasis* 2007;24:657–672. [PubMed: 18000759]
- Folling J, Bossi M, Bock H, Medda R, Wurm CA, Hein B, Jakobs S, Eggeling C, Hell SW. Fluorescence nanoscopy by ground-state depletion and single-molecule return. *Nat. Methods* 2008;5:943–945. [PubMed: 18794861]
- Fritz G, Brachetti C, Bahlmann F, Schmidt M, Kaina B. Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br. J. Cancer* 2002;87:635–644. [PubMed: 12237774]
- Garrett MD, Self AJ, van Oers C, Hall A. Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J. Biol. Chem* 1989;264:10–13. [PubMed: 2491843]
- Gomez del Pulgar T, Benitah SA, Valeron PF, Espina C, Lacal JC. Rho GTPase expression in tumorigenesis: evidence for a significant link. *BioEssays* 2005;27:602–613. [PubMed: 15892119]
- Gregory SL, Ebrahimi S, Milverton J, Jones WM, Bejsovec A, Saint R. Cell division requires a direct link between microtubule-bound RacGAP and Anillin in the contractile ring. *Curr. Biol* 2008;18:25–29. [PubMed: 18158242]

- Hara T, Abe M, Inoue H, Yu LR, Veenstra TD, Kang YH, Lee KS, Miki T. Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2/M phase. *Oncogene* 2006;25:566–578. [PubMed: 16170345]
- Heilemann M, van de Linde S, Schüttelz M, Kasper R, Seefeldt B, Mukherjee A, Tinnefeld P, Sauer M. Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. *Angew. Chem. Int. Ed* 2008;47:6172–6176.
- Hickson GR, O'Farrell PH. Anillin: a pivotal organizer of the cytokinetic machinery. *Biochem. Soc. Trans* 2008;36:439–441. [PubMed: 18481976]
- Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 1995;81:1159–1170. [PubMed: 7600583]
- Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol* 2005;21:247–269. [PubMed: 16212495]
- Kamai T, Arai K, Sumi S, Tsujii T, Honda M, Yamanishi T, Yoshida KI. The rho/rho-kinase pathway is involved in the progression of testicular germ cell tumour. *BJU Int* 2002;89:449–453. [PubMed: 11872041]
- Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Mol. Cell. Biol* 1995;15:6443–6453. [PubMed: 7565796]
- Kim J-E, Billadeau DD, Chen J. The tandem BRCT domains of Ect2 are required for both negative and positive regulation of Ect2 in cytokinesis. *J. Biol. Chem* 2005;280:5733–5739. [PubMed: 15545273]
- Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. *Am. J. Pathol* 2002;160:579–584. [PubMed: 11839578]
- Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol* 1996;16:5313–5327. [PubMed: 8816443]
- Lin R, Cerione RA, Manor D. Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Db1 transformation. *J. Biol. Chem* 1999;274:23633–23641. [PubMed: 10438546]
- Miki T, Smith CL, Long JE, Eva A, Fleming TP. Oncogene ect2 is related to regulators of small GTP-binding proteins. *Nature* 1993;362:462–465. [PubMed: 8464478]
- Molnar A, Theodoras AM, Zon LI, Kyriakis JM. Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. *J. Biol. Chem* 1997;272:13229–13235. [PubMed: 9148940]
- Muls B, Uji IH, Melnikov S, Moussa A, Verheijen W, Soumillion JP, Josemon J, Mullen K, Hofkens J. Direct measurement of the end-to-end distance of individual polyfluorene polymer chains. *ChemPhysChem* 2005;6:2286–2294. [PubMed: 16217812]
- Nishimura Y, Yonemura S. Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *J. Cell Sci* 2006;119:104–114. [PubMed: 16352658]
- Olson MF. Guanine nucleotide exchange factors for the Rho GTPases: a role in human disease? *J. Mol. Med* 1996;74:563–571. [PubMed: 8912177]
- Olson MF, Sahai E. The actin cytoskeleton in cancer cell motility. *Clin. Exp. Metastasis* 2009;26:273–287. [PubMed: 18498004]
- Paterson HF, Self AJ, Garrett MD, Just I, Aktories K, Hall A. Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J. Cell Biol* 1990;111:1001–1007. [PubMed: 2118140]
- Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 1993;90:8392–8396. [PubMed: 7690960]
- Piekny AJ, Glotzer M. Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis. *Curr. Biol* 2008;18:30–36. [PubMed: 18158243]
- Piekny A, Werner M, Glotzer M. Cytokinesis: welcome to the Rho zone. *Trends Cell Biol* 2005;15:651–658. [PubMed: 16243528]
- Qiu RG, Chen J, McCormick F, Symons M. A role for Rho in Ras transformation. *Proc. Natl. Acad. Sci. USA* 1995;92:11781–11785. [PubMed: 8524848]

- Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992;70:389–399. [PubMed: 1643657]
- Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat. Rev. Mol. Cell Biol* 2003;4:446–456. [PubMed: 12778124]
- Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol* 2005;6:167–180. [PubMed: 15688002]
- Sahai E, Marshall CJ. RHO-GTPases and cancer. *Nat. Rev. Cancer* 2002;2:133–142. [PubMed: 12635176]
- Sahai E, Ishizaki T, Narumiya S, Treisman R. Transformation mediated by RhoA requires activity of ROCK kinases. *Curr. Biol* 1999;9:136–145. [PubMed: 10021386]
- Saito S, Tatsumoto T, Lorenzi MV, Chedid M, Kapoor V, Sakata H, Rubin J, Miki T. Rho exchange factor ECT2 is induced by growth factors and regulates cytokinesis through the N-terminal cell cycle regulator-related domains. *J. Cell Biochem* 2003;90:819–836. [PubMed: 14587037]
- Self AJ, Paterson HF, Hall A. Different structural organization of Ras and Rho effector domains. *Oncogene* 1993;8:655–661. [PubMed: 8437849]
- Straight AF, Field CM, Mitchison TJ. Anillin binds nonmuscle myosin II and regulates the contractile ring. *Mol. Biol. Cell* 2005;16:193–201. [PubMed: 15496454]
- Takaishi K, Sasaki T, Kameyama T, Tsukita S, Takai Y. Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. *Oncogene* 1995;11:39–48. [PubMed: 7624130]
- Tatsumoto T, Xie X, Blumenthal R, Okamoto I, Miki T. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J. Cell Biol* 1999;147:921–928. [PubMed: 10579713]
- Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990–994. [PubMed: 9353125]
- Villanueva J, Yung Y, Walker JL, Assoian RK. ERK activity and G1 phase progression: identifying dispensable versus essential activities and primary versus secondary targets. *Mol. Biol. Cell* 2007;18:1457–1463. [PubMed: 17314399]
- Weber JD, Raben DM, Phillips PJ, Baldassare JJ. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem. J* 1997;326:61–68. [PubMed: 9337851]
- Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr. Biol* 2006;16:1171–1182. [PubMed: 16782007]
- Yonemura S, Hirao-Minakuchi K, Nishimura Y. Rho localization in cells and tissues. *Exp. Cell Res* 2004;295:300–314. [PubMed: 15093731]
- Yuce O, Piekny A, Glotzer M. An ECT2-centralspindlin complex regulates the localization and function of RhoA. *J. Cell Biol* 2005;170:571–582. [PubMed: 16103226]
- Zhao WM, Fang G. Anillin is a substrate of anaphase-promoting complex/cyclosome (APC/C) that controls spatial contractility of myosin during late cytokinesis. *J. Biol. Chem* 2005a;280:33516–33524. [PubMed: 16040610]
- Zhao WM, Fang G. MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. *Proc. Natl. Acad. Sci. USA* 2005b;102:13158–13163. [PubMed: 16129829]

Fig. 1. Inducible expression of V14RhoA results in myosin light chain (MLC) phosphorylation and actin stress fibre formation. (a) Uniformity of V14RhoA expression in V14RhoA-Swiss3T3 cells incubated in the absence or in the presence of tetracycline for 24 h was monitored by immunofluorescence analysis using anti-myc tag antibody. Nuclear DNA was stained with DAPI (blue). (b) V14RhoA-Swiss3T3 cells were serum-starved for 24 h in the absence or in the presence of tetracycline, with or without 10 μ M Y-27632, and then fixed and stained for F-actin (green) and phospho-MLC (red). Co-localization of red and green immunofluorescence is visualized as yellow. Nuclear DNA was stained with DAPI (blue). Bars: 100 μ m (a), 20 μ m (b).

Fig. 2. V14RhoA induction inhibits cell proliferation. (a) V14RhoA-Swiss3T3 cell number was determined 24, 48 or 72 h after incubation in the absence or in the presence of tetracycline, with or without Y-27632 (10 μ M), as indicated. Each condition was carried out in triplicate and expressed as mean $\pm$ standard error. Results of a representative experiment of two independent determinations are shown. (b) V14RhoA-Swiss3T3 or WTRhoA-Swiss3T3 cells were incubated in the absence or in the presence of different concentrations of tetracycline (ranging from 3.5 to 1000 ng/ml) for 24 or 48 h. V14RhoA and WTRhoA expression was monitored by Western blot using anti-myc tag primary antibody and near-infrared fluorescently labelled secondary antibody, quantified with a LI-COR Odyssey instrument and normalized

to ERK2 expression levels. Rho expression level was normalized to the condition that gave the lowest expression = 1. (c) The number of cells in each treatment group detailed above was determined. Each condition was carried out in triplicate and expressed as mean $\pm$ standard error. Results of a representative experiment of two independent determinations are shown.

Fig. 3. V14RhoA induction slows down serum-stimulated S-phase entry and blocks cells in G₂/M. (a) V14RhoA-Swiss3T3 cells were serum-starved for 24 h, in the absence or in the presence of tetracycline, and then serum-stimulated for 16 or 24 h in the absence or in the presence of tetracycline as indicated. Cells were analyzed by FACS as described in Materials and methods. Panels show cell numbers versus propidium iodide staining to quantify DNA content. Data of a representative experiment of 5 determinations with similar results are shown. (b) The distribution of cells in each cell cycle phase after initial serum starvation and after the additional 16 or 24 h serum stimulation was determined and expressed as a mean $\pm$ standard error (n=5 independent experiments; * p<0.05. ** p<0.01.).

Fig. 4. V14RhoA inhibits sustained phase of serum-induced MAPK activation. (a) V14RhoA-Swiss3T3 cells were serum starved for 24 h, in the absence or in the presence of tetracycline, and then serum stimulated for 1, 3, 6, 9 or 13 h in the absence or in the presence of tetracycline as indicated. Whole cell lysates were prepared and analyzed by Western blotting using antibodies directed against the V14RhoA myc-tag, phospho-ERK1/2 (Thr202/Tyr204), ERK2, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-Raf1 (S338) and Raf1. Results from a representative experiment of 4 determinations with similar results are shown. (b) Phospho-ERK1/2, phospho-MEK1/2 and phospho-Raf1 levels were detected using near-infrared fluorescently labelled secondary antibodies and quantified with a LI-COR Odyssey instrument,

and normalized to ERK2, MEK1/2 and Raf1 levels, respectively, and expressed as a mean $\pm$ standard error (at least 5 independent experiments; * $p < 0.01$) (c) V14RhoA-Swiss3T3 cells were serum starved for 24 h, in the absence or in the presence of tetracycline, and then serum stimulated for 5, 15, 30, 60 or 180 minutes in the absence or in the presence of tetracycline as indicated. Whole cell lysates were prepared and analyzed by Western blotting using antibodies directed against the V14RhoA myc-tag, phospho-ERK1/2 (Thr202/Tyr204), ERK2, phospho-MEK1/2 (Ser217/221) and MEK1/2. Results from a representative experiment of 4 determinations with similar results are shown. (d) Phospho-ERK1/2 levels were detected using near-infrared fluorescently labelled secondary antibodies and quantified with a LI-COR Odyssey instrument, and normalized to ERK2. The phospho-ERK/ERK ratio is expressed as a mean $\pm$ standard error (n=4 independent experiments; * $p < 0.01$). (e) V14RhoA-Swiss 3T3 cells were serum-starved for 24 h, in the absence or in the presence of tetracycline, and then serum stimulated for 1, 3, 6, 9 or 13 h in the absence or in the presence of tetracycline as indicated. Expression levels of V14RhoA, cyclin D1 and GAPDH mRNA were determined by real time PCR experiments as described in Materials and methods. Each condition was carried out in triplicate and repeated with 5 independent mRNA preparations. The relative differences of expression between each condition were expressed as mean $\pm$ standard error normalized to GAPDH expression levels at each time point (n=5 independent experiments).

Fig. 5.

V14RhoA induces formation of binucleate cells. (a) V14RhoA-Swiss3T3 cells were incubated in the absence or in the presence of tetracycline with Y-27632 (10 μ M) for 24 or 48 h and then fixed and stained for F-actin (green) and V14RhoA myc-tag (red). Nuclear DNA was stained with DAPI (blue). Results of a representative experiment obtained 48 h after tetracycline withdrawal are shown (left panel). Binuclear cells indicated by arrows. Scale bar, 50 μ m. The percentage of binucleate cells in each condition was determined and expressed as a mean $\pm$ standard error (n=3 independent experiments; right panel). (b) V14RhoA-Swiss3T3 cells were incubated in the absence or in the presence of tetracycline for 48 h. Images were taken every 10 min by time-lapse microscopy. A set of pictures showing a representative cytokinesis event

observed in the presence of tetracycline is shown (upper panels). The second and third sets of pictures show early and late cytokinesis defects observed in the absence of tetracycline (middle and lower panels, respectively).

Fig. 6. V14RhoA represses G₂/M regulatory proteins and fails to accumulate to the midbody. (a) V14RhoA-Swiss3T3 cells were incubated in the absence or in the presence of tetracycline for 24 h and then serum stimulated for 16 to 23 h. Whole cell extracts were analyzed by Western blot using antibodies directed against the V14RhoA myc-tag, anillin, ECT2 and cyclin B1. ERK2 was used as a loading control. (b) V14 or WTRhoA-Swiss3T3 cells were incubated in the presence (no induction) or absence of tetracycline for 24 h, and then fixed and stained for α -tubulin (red) and RhoA (green). Nuclear DNA was stained with DAPI (blue). Representative pictures of cells during metaphase, anaphase and telophase in each condition are shown. (c) Conventional wide-field fluorescence images of α -tubulin-FITC (green) and Rho-ACy5 (red)

during telophase, and super-resolution reconstructed image (SRI) of RhoA-Cy5 (corresponding to the boxed area in the conventional RhoA image). Scale bars, 10 μm (b), 500 nm (c).

Table 1

Primers for RT-PCR

Primer name	Sequence
RHOA-6S	TAGC GGATCC GCCACCATGGAGCAGAAGCTG
RHOA-7A	CATGATA AAGCTT TTCACAAGACAAGGCACCC
MutRHOA-2A	GCTCAGGGCGCCATCACCACAATCACCAGT
MutRHO-1S	ACTACGGGCGCCTGTGGAAAGACATGCTTGC
MuGAPDH	ACCACAGTCCATGCCATCA
MuGAPDH-2A	TCCACCACCCTGTTGCTGTA
RhoA-1S	GCCACCATGGAGCAGAAGCTG
RhoA-2A	CCTCGATA TCTGCCACATAG
MucyD-1S	TACCGCACACGCACTTTC
MucyD-2A	TGTCCACATCTCGCACGTC

BamHI and HindIII restriction sites are indicated in bold. NarI sites are underlined.