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Review

Controlling the switches: Rho GTPase regulation during animal cell mitosis

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ABSTRACT

Animal cell division is a fundamental process that requires complex changes in cytoskeletal organization and function. Aberrant cell division often has disastrous consequences for the cell and can lead to cell senescence, neoplastic transformation or death. As important regulators of the actin cytoskeleton, Rho GTPases play major roles in regulating many aspects of mitosis and cytokinesis. These include centrosome duplication and separation, generation of cortical rigidity, microtubule–kinetochore stabilization, cleavage furrow formation, contractile ring formation and constriction, and abscission. The ability of Rho proteins to function as regulators of cell division depends on their ability to cycle between their active, GTP-bound and inactive, GDP-bound states. However, Rho proteins are inherently inefficient at fulfilling this cycle and require the actions of regulatory proteins that enhance GTP binding (RhoGEFs), stimulate GTPase activity (RhoGAPs), and sequester inactive Rho proteins in the cytosol (RhoGDIs). The roles of these regulatory proteins in controlling cell division are an area of active investigation. In this review we will delineate the current state of knowledge of how specific RhoGEFs, RhoGAPs and RhoGDIs control mitosis and cytokinesis, and highlight the mechanisms by which their functions are controlled.

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1. Introduction

Since the initial description of roles for Rho proteins in cytokinesis in the mid-1990s, many labs have focused on characterizing the molecular mechanisms by which particular Rho proteins and their effectors regulate mitosis and cytokinesis. These efforts have shown that Rho GTPases control critical events pertaining to cell division including centrosome separation, spindle–kinetochore attachments, cytokinesis and abscission. In light of this knowledge, an important question yet to be fully answered is how the activities of Rho proteins are controlled in a temporal and spatial manner to ensure accurate cell division.

Rho GTPases are small GTP binding proteins that function as molecular switches, cycling between their active, GTP-bound and inactive GDP-bound states. Once active they initiate intracellular signaling pathways that control many cell functions, including mitosis and cytokinesis. As a general rule Rho proteins are inefficient GTPases, with catalytic activities far less than one mole of GTP hydrolyzed per minute *in vitro*. Moreover, Rho proteins are exceedingly slow in releasing GDP once hydrolysis has occurred [33,57,74]. Thus, to make Rho proteins workable as intracellular signaling enzymes, they require the aid of numerous regulators. These regulators fall into three classes, namely guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). RhoGEFs stimulate the release of GDP to enable GTP binding, thereby allowing Rho proteins to initiate intracellular signaling. RhoGAPs stimulate the intrinsic GTPase activity of Rho proteins, thereby turning off their signaling functions. RhoGDIs bind to inactive Rho proteins and sequester them in the cytosol. In some cases RhoGDIs also protect Rho proteins from degradation [15]. There are over 80 RhoGEFs in the human genome, nearly 70 RhoGAPs, and three RhoGDIs, and most cells express multiple members of each family [25,56,75,89]. Thus, a significant challenge in the Rho GTPase field is to understand which RhoGEFs and RhoGAPs participate in the regulation of particular cellular events. In the fields of mitosis and cytokinesis our understanding of the contributions of different Rho regulatory proteins is still a work in progress. In this review we will address the current understanding of the role of these regulatory proteins in animal cell division.

2. Rho GTPase function in mitosis and cytokinesis

There are over 20 Rho proteins in the human genome [37]. However, only three of these, Cdc42, Rac1 and RhoA, are widely recognized to contribute to mitosis and cell division (Fig. 1). Moreover, their individual contributions can vary depending on the cell type being assessed. To better understand the role of Rho regulatory proteins in mitosis and cytokinesis, we will briefly review the evidence directly implicating Rho proteins in these processes.

2.1. Cell division functions of Cdc42 and Rac1

Cdc42 was first shown to impact cell division in fertilized *Xenopus laevis* embryos, where injection of either recombinant, constitutively active V^{12}Cdc42 or dominant negative N^{17}Cdc42 inhibited cytokinesis [26]. This phenotype was more penetrant for V^{12}Cdc42 and was typified by a regression of the cleavage furrow after initial specification. When the embryos were sectioned and stained for F-actin it was evident that cortical F-actin remained, but that contractile ring formation was impaired. Concurrent with this work, another group showed that inducible expression of V^{12}Cdc42 in HeLa cells caused the accumulation of giant, multinucleate cells, suggesting that cytokinesis was disrupted [27]. Using a Raichu FRET reporter probe it was shown a few years later that Cdc42 activity was low throughout mitosis until cytokinesis, at which time Cdc42 activation was detected on intracellular membranes [100]. Many years later it was shown by RNAi in NRK cells that knockdown of Cdc42 interfered with F-actin accumulation at the cleavage furrow, further suggesting a role during cytokinesis [105]. However, a mechanistic role for Cdc42 during cytokinesis has yet to be defined.

A more distinct role for Cdc42 has been demonstrated earlier in mitosis. The first hint of such a role came from experiments demonstrating that Toxin B treatment of HeLa cells, which blocks all Rho GTPases, strongly inhibited chromosome alignment during metaphase [97]. This resulted in Mad-2 localization to kinetochores, which indicated that the spindle assembly checkpoint was activated. Expression of dominant negative Cdc42, but not dominant negative Rac1 or RhoA, caused a similar defect and resulted in the propagation of cells with irregularly shaped nuclei and micronuclei. Both of these phenotypes are consistent with chromosome congression defects. Moreover, the Cdc42 effector mDia3 was localized to kinetochores and interacted with the centromeric histone CENP-A. Colocalization of mDia3 with CENP-A was disrupted by toxin B treatment, indicating that endogenous Rho GTPase activity was required for kinetochore localization. Importantly, transfection of siRNA targeting mDia3, but not the related formin mDia1, also caused chromosome misalignment during metaphase [97].

In later work it was shown that the RhoGEF Ect2 was responsible for Cdc42 activation during chromosome congression [70]. Using an RBD pulldown assay, Cdc42 activity was found to be high in G2, low in pro-metaphase, high in metaphase and low again in telophase. In addition, transfection of siRNAs specific for Ect2, or expression of the Ect2 N-terminus, blocked metaphase activation of Cdc42. Interestingly,

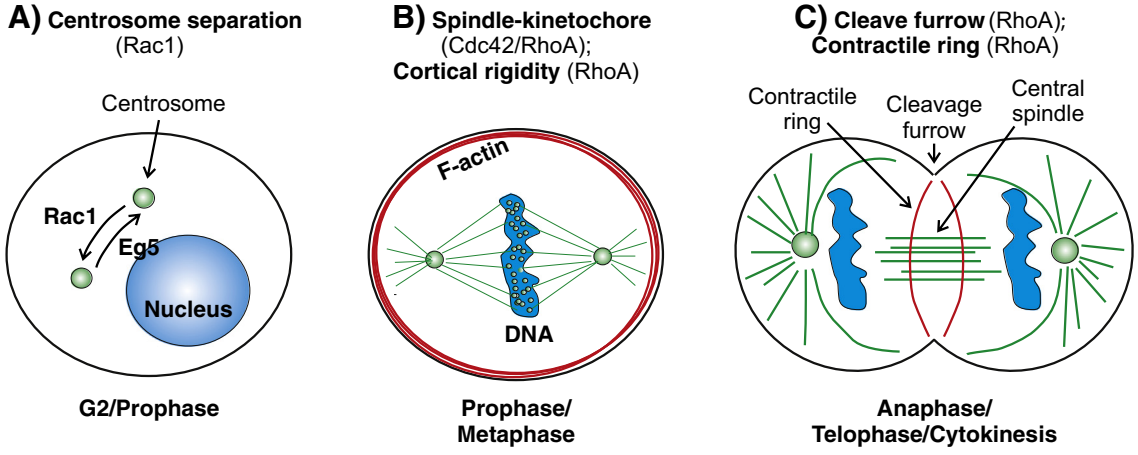

Fig. 1. Roles of Rho GTPases in mitosis and cytokinesis. A) Centrosome separation. Centrosome separation and convergence forces are denoted with arrows. Occurs in G2 and prophase. B) Mitotic spindle–kinetochore attachment stability and generation of cortical rigidity. Cortical F-actin is shown in red. Kinetochores are shown as green circles within condensed DNA. Occurs in prophase through metaphase. C) Cleavage furrow formation; contraction ring formation and constriction. Occurs during anaphase, telophase and cytokinesis.

expression of catalytically-inactive MgcRacGAP/hsCYK-4, which is a RhoGAP with a well-established role in cytokinesis, also caused the accumulation of cells with misshapen or micronuclei, suggesting that it may also play a role in regulating Cdc42 function during chromosome alignment. An important point in this work was that Cdc42 siRNA transfection was far less effective at causing chromosome misalignment than expression of dominant negative Cdc42, indicating that other Rho GTPases may regulate microtubule attachment to kinetochores. This mystery was later resolved when it was shown that expression of the Cdc42 related GTPases TC10, TCL, Wrch1 and Wrch2 were also required for chromosome alignment, although their contribution was clearly less important than Cdc42 [98].

Distinct from Cdc42, little evidence indicates a mitotic role for Rac1. In fact, it is clear that Rac1 activity is suppressed during mitosis [100], and that failure to inhibit Rac1 activation during mitosis results in a cytokinesis defect characterized by inappropriate cell spreading and adhesion [10]. This work supports the long held observation that expression of constitutively active Rac1 causes the formation of multinucleate cells, which is indicative of a cytokinetic defect [65]. However, recent work suggests that Rac1 may function early in prophase to control the rate of spindle pole separation. In this work it was demonstrated that Rac1 and its GEF Tiam1 localize to spindle poles in MDCK II cells, and that siRNA to Tiam1 causes the spindle poles to move farther apart during prophase [94]. This phenotype was counteracted by treatment with low doses of the Eg5 inhibitor monastrol, which slowed the rate of spindle pole separation. Tiam1 siRNA or treatment with the Rac1 inhibitor NSC23766 caused a delay in pro-metaphase and an increase in chromosome congression defects, indicating that the delay in spindle pole separation impacted later mitotic events.

2.2. Cell division functions of RhoA

RhoA was recognized to contribute to mitotic progression when it was discovered that injection of C3 exoenzyme from *Clostridium botulinum*, which ADP ribosylates and inactivates RhoA, RhoB and RhoC, prevented cleavage furrow formation and cell division in *Xenopus* embryos and sand dollar eggs [43,50]. The inspiration for these experiments was prior work showing that RhoA controlled actomyosin contraction in interphase cells [72]. Since constriction of the F-actin containing contractile ring was known to be essential for cytokinesis, it seemed that RhoA was a likely candidate to control this activity. It was later shown that C3 treatment also caused ADP ribosylation of RhoA and prevented cell division in human hematopoietic cell lines [1]. It was apparent that precise control of the timing of RhoA activation was important when it was shown that injection of recombinant constitutively active RhoA, as well as the RhoA inactivating exoenzyme C3, just prior to cytokinesis blocked cleavage furrow formation in *Xenopus* embyros. Importantly, both treatments prevented F-actin accumulation at the cleavage furrow, but did not affect insertion of new membrane into the nascent cleavage furrow [26]. Thus, it became clear that RhoA activity was necessary for assembly of the actin contractile ring.

Later experiments established that RhoA was activated between prophase and metaphase, and also in late telophase in HeLa cells [42,100]. Interestingly, RhoA activation could be blocked by expression of the N-terminal regulatory domain of the RhoGEF Ect2, suggesting that Ect2 regulated RhoA activity during mitosis [42]. Although early experiments showed that overexpressed RhoA localized to the cleavage furrow [87], this observation was not replicated until an uncommon TCA fixation protocol was utilized [99,102]. At the same time, using a GFP-RBD reporter it was shown in echinoderm and *Xenopus* eggs that RhoA activation preceded cleavage furrow ingression and was dependent upon microtubule assembly, but did not require F-actin polymerization [11]. This group also demonstrated that physical movement of the central spindle displaced the RhoA activation zone in the direction of spindle movement, thus confirming that the central spindle coordinates RhoA activity.

In aggregate these results indicate that RhoA activation is essential for cytokinesis in most cell types. However, an interesting wrinkle on this theme arose from observations that some mammalian cells do not require RhoA for cytokinesis. For example, it was shown that C3 exoenzyme did not block cytokinesis in mouse 3T3 fibroblasts, and only prevented cytokinesis about half of the time in NRK cells [69]. These authors noted, however, that inhibition of RhoA caused a significant distortion of the midzone membrane in NRK cells and promoted ectopic cleavage furrow formation. This was also accompanied by reduced F-actin and myosin II staining in the membrane at the site of the developing cleavage furrow. These observations prompted the authors to posit that RhoA was only necessary for cytokinesis in cells that were poorly adherent during mitosis, and that in adherent cells its main role was to maintain cortical membrane rigidity. Results such as these were later extended when it was shown that Rat1A and NIH3T3 cells did not require RhoA for cytokinesis [101]. It was noted, however, that actomyosin contraction was still required for cytokinesis in cell lines that did not need RhoA, as treatment with the myosin inhibitor blebbistatin prevented cytokinesis in these cells. Thus, the inference was that RhoA-independent cell lines utilized a distinct mechanism to control actomyosin contraction in the cleavage furrow. These authors also observed that it was important to suppress Rac1 activation during mitosis in all cell types, and that expression of constitutively active Rac1 or inhibition of MgcRacGAP/hsCYK-4 caused a penetrant defect in cytokinesis.

Evidence that RhoA controls cortical rigidity during mitosis was presented by Maddox and Burridge when they demonstrated that C3 exoenzyme treatment prevented HeLa cell rounding and suppressed cortical rigidity [52]. They also demonstrated that RhoA accomplished this through activation of its effector ROCK, as ROCK inhibition with Y-27632 phenocopied C3 exoenzyme treatment. The authors also demonstrated that the RhoA GAP p190-RhoGAP was activated during mitosis, but did not regulate cell rounding.

3. RhoGEFs in mitosis and cytokinesis

3.1. RhoGEFs in mitosis

RhoGEFs have been shown to contribute to a number of processes during mitosis, including centrosome duplication (ARHGEF10), centrosome separation (Tiam1), and the formation of microtubule–kinetochore attachments (Ect2, GEF-H1, and Net1) (Fig. 2). Roles in stabilizing microtubule–kinetochore attachments are somewhat complicated by variable requirements for Rho GTPase function among different cell lines.

3.1.1. ARHGEF10

ARHGEF10 is a relatively understudied RhoA GEF that appears to play a critical role in controlling centrosome duplication. ARHGEF10 was shown to localize to centrosomes in HeLa cells, and siRNA knockdown of ARHGEF10 in HeLa or U2OS cells resulted in the accumulation of supernumerary centrosomes. This, in turn, resulted in the appearance of multipolar spindles [2]. Importantly, ARHGEF10 knockdown did not cause the accumulation of multinucleate cells, indicating that it did not contribute to cytokinesis. The authors also reported that RhoA knockdown caused the accumulation of supernumerary centrosomes in HeLa or U2OS cells, something that has not been reported by others. Moreover, expression of constitutively active RhoA restored centrosome regulation in ARHGEF10 knockdown cells, supporting a role for RhoA in control of centrosome duplication. ARHGEF10/RhoA control of centrosome duplication may occur through ROCK activation, as treatment of cells with the ROCK inhibitor Y-27632 also caused supernumerary centrosomes. This finding is supported by a previous study indicating that ROCKII regulates centrosome duplication [49]. The authors also found that ARHGEF10 interacted with the kinesin motor protein KIF3B, which is a component of the kinesin-2 complex. KIF3B has been

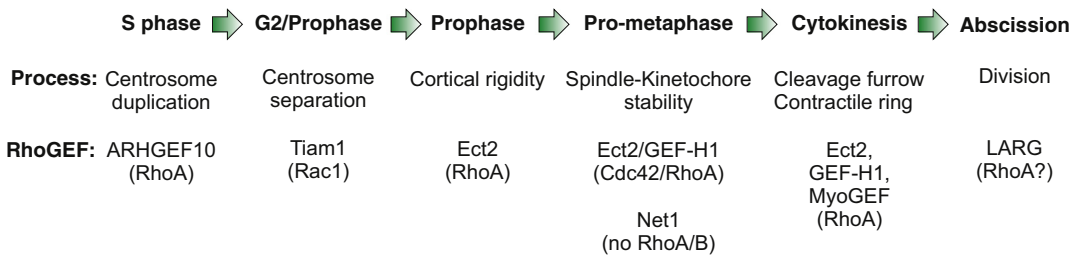

Fig. 2. RhoGEFs in mitosis and cytokinesis.

shown previously to interact with the kinesin-2 complex components KIF3A and KAP3, and to localize to centrosomes and the mitotic spindle. In addition, expression of a KIF3B mutant that cannot interact with KAP3 caused the accumulation of extra centrosomes and abnormal spindle formation in NIH3T3 cells [32]. Aoki et al. demonstrated that transfected KIF3B localized to centrosomes in HeLa cells, and that siRNA knockdown of KIF3B resulted in the appearance of supernumerary centrosomes [2]. However, the specific function of KIF3B in centrosome duplication, and the role of ARHGEF10/RhoA/ROCK in this regulation are unclear.

3.1.2. Tiam1

A role for the Rac1-specific GEF Tiam1 in controlling centrosome separation during mitosis has recently been established. Endogenous Tiam1 and Rac1 localize to centrosomes during prophase and pro-metaphase in MDCK II cells, and inhibition of their expression speeds the separation of centrosomes during early mitosis [94]. This, in turn, results in aberrant congression of chromosomes during metaphase. Centrosome separation prior to nuclear envelope breakdown is known to be driven by the kinesin motor protein Eg5, and inhibition of Eg5 results in monopolar mitotic spindles [14,34]. Treatment of cells with low doses of the Eg5 inhibitor STLC that partially blocks Eg5 function results in slower separation of centrosomes. Importantly, this can be rescued by Tiam1 siRNA transfection or by concurrent treatment with the Rac1 inhibitor NSC23766 [94]. Thus, Tiam1 acts through Rac1 to balance Eg5 function and control the timing of centrosome separation during prophase and pro-metaphase.

3.1.3. Ect2

Ect2 is an efficient GEF for Rac1, Cdc42 and RhoA, and is an essential gene in mice [22,88]. As mentioned earlier, Ect2 function is important for kinetochore attachment in many cell types. This was first recognized in HeLa cells, where it was shown that Ect2 knockdown inhibited chromosome alignment during metaphase and resulted in activation of the spindle assembly checkpoint [70]. This caused the appearance of lagging chromosomes during anaphase, ultimately promoting the accumulation of abnormally shaped nuclei and micronuclei in interphase cells. Distinct from its role in cytokinesis, Ect2 knockdown inhibited Cdc42 activation during pro-metaphase and reduced the localization of CENP-A within centrosomes.

Another important role for Ect2 during mitosis is to regulate cortical rigidity of the plasma membrane. Mitotic cells generally undergo profound changes in cell shape that begin as they exit G2. This requires dramatic, RhoA-dependent changes in actin cytoskeletal organization, and results in a substantial increase in cortical rigidity [52]. Recently it was shown that knockdown of Ect2 in HeLa cells delayed mitotic cell rounding and blocked cortical accumulation of F-actin [55]. Ect2 knockdown also prevented the increase in cortical rigidity characteristic of mitotic cells. The ability of Ect2 to control mitotic F-actin accumulation required nuclear export during prophase, and re-import of Ect2 into the nucleus during late telophase terminated cortical RhoA activation [55].

3.1.4. GEF-H1/LFC

In cells that do not require RhoA for cytokinesis, such as Rat2 fibroblasts, NIH3T3 fibroblasts and Ptk rat kidney epithelial cells, GEF-H1 (also known as *Lfc* in the mouse) is required for spindle assembly during pro-metaphase. This was shown by injection of anti-Lfc antibodies, as well as by RNAi. In these cells GEF-H1 controlled the attachment of spindle microtubules to kinetochores, but importantly, did not play a role in cytokinesis [8]. In addition, spindle assembly was shown to be RhoA subfamily-dependent, as injection of cells with C3 toxin blocked spindle assembly. Furthermore, this required the function of the RhoA effector mDia1, which unlike mDia3 is regulated only by RhoA [8]. These data indicate that in cells that do not require RhoA for cytokinesis, such as Rat2 cells, kinetochore attachment is controlled through a GEF-H1/RhoA/mDia1 pathway. However, in cells that do require RhoA for cytokinesis, kinetochore attachment would be controlled through an Ect2/Cdc42/mDia3 pathway [97].

3.1.5. Net1

The RhoA/RhoB-specific GEF Net1 was recently shown to be important for kinetochore attachment to centrosomes and chromosome congression during metaphase. There are two isoforms of Net1 in most cells, Net1 and Net1A, and overexpression of either isoform in HeLa cells caused abnormal nuclear morphologies indicative of errors in mitotic progression [58]. However, overexpressed Net1 was more effective than Net1A at causing mitotic defects, and knockdown of Net1, but not Net1A, caused the accumulation of aberrant nuclei. Thus, the Net1 isoform appears to play a predominant role in mitosis. This fits with separate studies indicating that the Net1 isoform is required for proliferation in MCF7 cells [28], and that Net1A primarily regulates cell motility [19,20,47,66]. Live cell imaging indicated that Net1 expression was important for progression from prophase to anaphase, as depletion of Net1 dramatically lengthened the time required for transit of these phases of mitosis. However, once cells entered anaphase they no longer required Net1 expression, as there was no effect of Net1 knockdown on the anaphase to telophase transition. Net1 may contribute to kinetochore attachment to the centrosomes, as Net1 knockdown resulted in reduced cold stability of the metaphase spindle and caused activation of the spindle assembly checkpoint. Intriguingly, the ability of Net1 to control this process did not require Rho GTPase activation, as depletion of RhoA or RhoB did not phenocopy the effects of Net1 knockdown, and expression of a catalytically-inactive version of Net1 effectively rescued mitosis in Net1 knockdown cells [58]. A clue as to how Net1 may control mitosis came from the observation that loss of Net1 expression prevented activation of Aurora A and its regulatory kinase Pak2 at the centrosome. As Aurora A function is required for activation of the spindle assembly factors such as TACC3, this suggests that Net1 may indirectly affect spindle assembly dynamics through regulation of Aurora A activation.

3.2. RhoGEFs in cytokinesis

Work from a number of groups indicates that at least four RhoGEFs control RhoA activation during cytokinesis, including Ect2, GEF-H1, MyoGEF and LARG (Fig. 2). Moreover, these proteins appear to be

sequentially activated as cleavage furrow ingression progresses. Ect2 is essential for initiation of cleavage furrow ingression, and as furrow ingression progresses GEF-H1 and MyoGEF activation occur concurrent with Ect2 activation. Lastly, activation of LARG is required for abscission, which is the final step of cytokinesis.

3.2.1. Ect2

Ect2 was first recognized to contribute to cell division when it was shown that expression of the N-terminal regulatory domain of Ect2, or microinjection of an antibody to Ect2, blocked cytokinesis. In this work Ect2 was also shown to localize to the mitotic spindle and the midbody [88]. About this time another group showed that overexpression of the Ect2 N-terminus blocked the increase in RhoA GEF activity that is normally observed within lysates derived from cells in telophase [42]. Confirmation of the role of Ect2 in cytokinesis was later demonstrated using siRNA transfection approaches [21,41]. Ect2 (known as Pebble in *Drosophila melanogaster*) is recruited to the mitotic spindle during anaphase by interaction with the centralspindlin complex [40,68,81,102,104]. Centralspindlin is a heterodimer of the RhoGAP hsCYK-4/MgcRacGAP and the kinesin-like protein MKLP-1 [38]. Interaction of Ect2 with centralspindlin is necessary for cytokinesis in most animal cells. Inhibition of this interaction prevents accumulation of RhoA, F-actin, phospho-myosin light chain, and anillin at the cortical membrane adjacent to the central spindle, all of which are necessary for initiation and ingression of the cleavage furrow [5,44,71,78].

Regulation of Ect2 activity during cytokinesis is complex and not fully understood. Like many RhoGEFs, the N-terminus of Ect2 appears to function as an autoinhibitory domain. This is based on work showing that the N-terminus of Ect2 can interact with the C-terminus, which contains the catalytic DH/PH domains, and block its GEF activity [41]. Interestingly, the ability of the N-terminus to inhibit cytokinesis depends on the presence of the second of two BRCT domains. BRCT domains are phospho-motif recognition domains, suggesting that the Ect2 N-terminus blocks cytokinesis by preventing interaction of endogenous Ect2 with one or more phosphorylated proteins during cytokinesis. Moreover, Ect2 containing a point mutation in its second BRCT domain is unable to rescue cytokinesis in cells transfected with Ect2 siRNA [41]. Importantly, these BRCT domains are necessary for Ect2 recruitment to the central spindle, which occurs *via* interaction with the hsCYK-4 component of the centralspindlin complex. This is mediated by phosphorylation of the hsCYK-4 by Plk1, which creates a binding site for the BRCT domains of Ect2 [17,93]. This most likely activates Ect2 within the spindle, as it would be expected to prevent autoinhibition of Ect2 by its N-terminus.

Ect2 must localize to the equatorial plasma membrane during anaphase to promote cytokinesis. The C-terminus of Ect2 is sufficient for plasma membrane recruitment, and this requires the PH domain and adjacent polybasic region (PBR) within the C-terminus [84]. Together these regions mediate binding to phosphoinositides such as PI-4P, $PI(4,5)P_2$ and $PI(3,4,5)P_3$. Interestingly, centralspindlin is also required for equatorial membrane recruitment. Both the catalytic activity and membrane recruitment of Ect2 are necessary for cleavage furrow formation, and mutation of either the DH domain or the PH/PBR region of Ect2 prevents RhoA activation and anillin accumulation at the cleavage furrow [84]. Anillin recruitment to the cleavage furrow also helps restrict Ect2 localization within the region of the central spindle. This is mediated by binding of anillin to the PH domain of Ect2, and can be disrupted by mutation of D668 within Ect2 [29]. Thus, anillin and Ect2 positively reinforce the recruitment of each protein to the central spindle and equatorial plasma membrane.

Mitotic activation of Ect2 depends on its phosphorylation, although the mechanism by which this occurs is not clear. Ect2 is highly phosphorylated during mitosis, and dephosphorylation of Ect2 immunoprecipitated from mitotic lysates inhibits its GEF activity *in vitro* [42,88]. Not surprisingly, Cdk1 is a potent Ect2 kinase which phosphorylates multiple sites, including T412, T431 and T815 [31,67,84]. There is conflicting data as to the overall effect of Ect2 phosphorylation by Cdk1. Phosphorylation of T412 may contribute to the GEF activity of Ect2, since a T412A mutant exhibits less RhoA GEF activity in interphase cells. Phosphorylation of T412 also creates a binding site for the polo box of Plk1 [67]. Paradoxically, phosphorylation of T341 by Cdk1 may inhibit Ect2 GEF activity, since substitution of this site with an aspartate increases the affinity of the N-terminus for the C-terminal domain [31]. Moreover, phosphorylation of this site likely inhibits recruitment of Ect2 to the central spindle, as mutation of this site to alanine allows the Ect2 N-terminus to co-immunoprecipitate with hsCYK-4 in transfected HeLa cells [102]. Phosphorylation of T815 may block the association of Ect2 with the plasma membrane, as substitution of T815 with alanine promotes the localization of the Ect2 C-terminus to the plasma membrane during mitosis. Similarly, inhibition of Cdk1 activity also promotes Ect2 C-terminal membrane localization [84]. Thus, on balance, phosphorylation by Cdk1 appears to inhibit Ect2 activity. This fits with the timing of RhoA activation during mitosis, which occurs after Cdk1 activity is inhibited during the metaphase to anaphase transition. Plk1 also phosphorylates Ect2, although the phosphorylation sites have yet to be identified and the functional significance of this phosphorylation is not yet clear [67].

As with many important mitotic regulators, Ect2 protein accumulates during mitosis and decreases again as cells enter telophase [48,76,88]. Interaction of Ect2 with the scaffold protein HEF1 during mitosis protects Ect2 from degradation, as overexpression of HEF1 prevents Ect2 degradation and siRNA mediated knockdown of HEF1 reduces Ect2 expression. Importantly, HEF1 expression is also required to maintain RhoA activation during cytokinesis [24]. Ect2 is subject to proteasome-mediated degradation by the APC/C complex, which degrades mitotic proteins such as cyclin B as cells enter anaphase (for reviews see [60,73]). Treatment of cells with the proteasome inhibitor MG132 prevents Ect2 degradation and allows for accumulation of slower mobility forms Ect2 that correlate with its phosphorylation [48]. Ect2 is ubiquitylated *via* a K11 linkage by Cdh1, which is one of the two ubiquitin E3 ligases associated with APC/C. Interestingly, targeting of Ect2 by Cdh1 requires a C-terminal sequence between amino acids 800-826 of Ect2, which contains two putative TEK boxes, a D-box and bipartite NLS sequences [48].

3.2.2. MyoGEF

MyoGEF was identified in a screen for GFP-tagged RhoGEFs that localize to the cleavage furrow in mitotic cells. It controls RhoA activation in cells, but does not regulate Rac1. Endogenous MyoGEF localizes to the mitotic spindle, spindle poles and cleavage furrow, and siRNA mediated knockdown of MyoGEF in U2OS cells results in multinucleation [95]. MyoGEF is phosphorylated on threonine 174 by Plk1, which stimulates MyoGEF localization to the spindle poles and central spindle. Phosphorylation of T174 by Plk1 is also required for maximal GEF activity towards RhoA *in vitro* and in mitotic cells [6]. Interaction of Plk1 with MyoGEF is controlled by phosphorylation on threonine 544 by Aurora B kinase, which creates a docking site for the polo box domains within Plk1. Endogenous Aurora B and MyoGEF co-localize at the central spindle and midbody, and inhibition of Aurora B activity blocks MyoGEF localization to the central spindle [96]. MyoGEF also interacts with the centrosome/spindle pole associated protein (CSPP), and depletion of CSPP blocks MyoGEF localization to the central spindle and inhibits cytokinesis. Interestingly, siRNA depletion of either MyoGEF or CSPP does not interfere with cleavage furrow initiation, but instead results in regression of the cleavage furrow midway through cytokinesis [7]. Since others have shown that Ect2 depletion prevents cleavage furrow ingression altogether [21,40,68,104], these data indicate that MyoGEF and Ect2 fulfill distinct roles during cytokinesis, with MyoGEF modifying the dynamics of cleavage furrow ingression rather than licensing its initiation. This may explain why the effect of Ect2 knockdown on cytokinesis is stronger than MyoGEF knockdown. Intriguingly, MyoGEF and Ect2 interact during

cytokinesis, and knockdown of MyoGEF causes Ect2 mislocalization from the midbody [7].

3.2.3. GEF-H1

GEF-H1 was first observed to localize to the mitotic spindle in MDCK cells [12], and was shortly thereafter shown to play a role in mitotic spindle assembly in Rat-2 cells, which do not require RhoA activation for cytokinesis [8]. Endogenous GEF-H1 was also shown to localize to the mitotic spindle during metaphase in HeLa cells, to a ring-like structure surrounding the cleavage furrow during cytokinesis, and to the midbody in late stage cytokinesis [13]. In HeLa cells, perturbation of GEF-H1 activity, either by siRNA or by overexpression of catalytically-inactive GEF-H1, caused the appearance of multinucleated cells, indicative of a cytokinesis defect. Live cell imaging revealed that GEF-H1 knockdown cells exhibited membrane blebs and ectopic cleavage furrows, suggesting that GEF-H1 was important for coordinating cortical membrane activity during cytokinesis. Using a FRET sensor to detect RhoA activation, it was apparent that GEF-H1 was required for RhoA activation in the cleavage furrow during ingression. This was distinct from the effect of Ect2 knockdown, which prevent RhoA activation at the equatorial membrane and inhibited cleavage furrow formation overall. GEF-H1 activity in mitotic HeLa cells is negatively regulated by phosphorylation by Aurora A kinase on S885 and by Cdk1 on S959. Phosphorylation of S885 by Aurora A kinase occurs at the spindle poles, promotes binding of 14-3-3, and also inhibits GEF-H1 activity towards RhoA. Dephosphorylation of GEF-H1 occurs at the metaphase-anaphase transition and results in an increase in GEF-H1 catalytic activity [13]. These data indicate that in cells which require RhoA activity for cytokinesis, GEF-H1 limits cells to a single cleavage furrow and is required for cleavage furrow ingression.

3.2.4. LARG

Recently the RhoGEF LARG was shown to be required for the last step of cytokinesis, namely abscission [54]. LARG localizes to the spindle poles and central spindle during mitosis, and to the midbody during cytokinesis. Interestingly, LARG does not seem to be required earlier in mitosis. siRNA depletion of LARG in HeLa cells results in cells with intercellular bridges that do not cleave, suggesting that abscission has been inhibited. Moreover, these cells often die by apoptosis. Inhibition of Aurora B kinase, which controls the abscission checkpoint [51], rescues LARG knockdown cells and prevents apoptosis. These data indicate that LARG expression is necessary for cell division. However, it is as yet unclear whether the RhoA activation by LARG is required for abscission.

4. RhoGAPs in mitosis and cytokinesis

As Rho GTPases require cycling between their GTP-bound and GDP-bound states to effectively control cell signaling, it is logical to assume that each of their cell division functions will be regulated by one or more GAPs. RhoGAPs that have been implicated in regulating mitosis include MP-GAP (cortical rigidity), hsCYK-4 and p190B-RhoGAP (microtubule–kinetochore stability). RhoGAPs implicated in controlling cytokinesis include hsCYK-4, p190A-RhoGAP and p50-RhoGAP (Fig. 3).

4.1. hsCYK-4

The most highly studied Rho regulatory protein in mitosis is the RhoGAP hsCYK-4/MgcRacGAP. CYK-4 was originally identified as necessary for cytokinesis through genetic analysis in *C. elegans* [38]. CYK-4 is the ortholog of human MgcRacGAP/hsCYK-4, and both CYK-4 and mouse MgcRacGAP are essential for viability [38,91]. *C. elegans* and human CYK-4 are efficient GAPs for Rac1 and Cdc42, but not RhoA *in vitro* [38,90]. Initial work indicated that CYK-4 localized to the central spindle in *C. elegans* embryos and loss of CYK-4 prevented cytokinesis by disrupting the central spindle [38]. Importantly, central spindle localization of CYK-4 was dependent upon interaction with the kinesin-like protein ZEN-4 [38]. Soon thereafter it was shown that overexpression of mouse MgcRacGAP in human HL-60 cells blocked proliferation due to a cytokinesis defect. Endogenous hsCYK-4 was shown to localize to the cleavage plane and midbody in HeLa cells, and overexpression of catalytically-inactive hsCYK-4 in HeLa cells caused multinucleation. hsCYK-4 was also shown to interact with all three tubulin isoforms *via* its N-terminal myosin-like domain [35]. Taken together this work convincingly demonstrated that CYK-4 expression is essential for cytokinesis.

CYK-4 and ZEN-4 (MKLP-1 in humans; Pavarotti in *Drosophila*) form a complex referred to as centralspindlin, which is composed of two subunits of each protein [62,81]. This interaction is essential for cytokinesis and is mediated by the coiled coil domain in CYK-4 and the neck/coiled coil regions in ZEN-4. The central spindle is composed of antiparallel microtubule bundles and, importantly, the centralspindlin complex is sufficient to induce microtubule bundling *in vitro* [62]. The interaction of centralspindlin with microtubules in cells is negatively regulated by phosphorylation by Cdk1. Cdk1 phosphorylates ZEN-4 on T9 and T450. T9 lies within an N-terminal extension that is essential for ZEN-4 motor activity and phosphorylation of this site potently inhibits the ability of ZEN-4 to bind to microtubules and hydrolyze ATP *in vitro* [63]. Thus, Cdk1 phosphorylation represents a mechanism to inhibit centralspindlin function prior to anaphase, after which Cdk1 is inactivated by degradation of cyclin B. In support of this, a non-phosphorylatable mutant of MKLP-1 expressed in HeLa cells mislocalizes to the metaphase spindle and causes the appearance of lagging chromosomes in anaphase [63].

As mentioned above, CYK-4 was characterized *in vitro* as an active GAP for Rac1 and Cdc42, but not RhoA. Surprisingly, an early study indicated that phosphorylation of hsCYK-4 by Aurora B kinase converted CYK-4 to a RhoA GAP at the expense of its Rac GAP activity, suggesting that the GAP activity of CYK-4 in cells was malleable [61]. However, no other RhoGAPs have been shown to be regulated in this manner, and this finding has since been disputed by other groups [10,18]. It is clear, though, that the Rac1/Cdc42 GAP activity of CYK-4 is essential for cytokinesis [18,35]. Expression of constitutively active Rac1 disrupts cytokinesis, and expression of catalytically-inactive hsCYK-4 in hsCYK-4 knockdown cells results in inappropriate Rac1 activation and cytokinesis failure. Moreover, cytokinesis failure induced by expression of constitutively active Rac1 can be rescued by siRNA to ARHGEF7/βPIX, or Pak1 and Pak2, which are Rac1 effectors required for cell adhesion [10]. Thus, it is likely that the GAP activity of hsCYK-4 is required to prevent

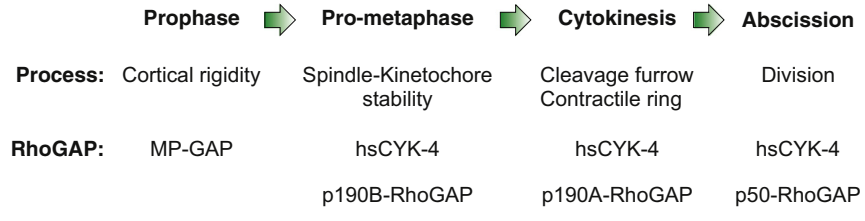

Fig. 3. RhoGAPs in mitosis and cytokinesis.

premature, Rac1-dependent cell spreading and adhesion during cytokinesis. It follows then that the timing of CYK-4 GAP activation is tightly regulated. For example, the mitotic spindle associated protein PRC1 binds to hsCYK-4 and inhibits its activity towards Cdc42 *in vitro*. This interaction is necessary for correct metaphase spindle formation, most likely due to the role of Cdc42 in promoting spindle attachment to kinetochores [70,97,98]. Phosphorylation of hsCYK-4 by Aurora B kinase inhibits this interaction, thus indirectly upregulating the Cdc42 GAP activity of hsCYK-4 [9]. The GAP activity of CYK-4 may also be inhibited during cytokinesis by interaction with SHCBP1. SHCBP1 is recruited to the midbody and overexpression of SHCBP1 disrupts cytokinesis. Importantly, the *Drosophila* homolog of SHCBP1, Nessun Dorma, is required for cytokinesis in male germ cells [64]. SHCBP1 inhibits the Rac1 GAP activity of hsCYK-4 *in vitro* and expression of dominant negative Rac1 rescues cleavage furrow formation in SHCBP1 overexpressing cells. In addition, phosphorylation of SHCBP1 by Plk1 prevents recruitment of SHCBP1 to the central spindle, indicating that the timing of SHCBP1 interaction with hsCYK-4 is precisely regulated [4].

An additional important function of hsCYK-4 is to control the recruitment of Ect2 to the central spindle, which is essential for cortical RhoA activation and cleavage furrow formation [40,68,102,104]. Ect2 is recruited to hsCYK-4 after hsCYK-4 phosphorylation by Plk1, which creates binding sites for the BRCT domains within Ect2 [17,93,102]. Plk1 phosphorylates four sites within hsCYK-4. Two of these, S157 and S170, are required for Ect2 recruitment. Interestingly, binding of PRC1 to hsCYK-4 facilitates hsCYK-4 phosphorylation by Plk1 [17].

hsCYK-4 may also cooperate with Ect2 to maintain newly deposited CENP-A within the centromere, which is required for mitotic spindle assembly and chromosome segregation. CENP-A is a histone variant that is an essential component of the centromere, and during DNA replication each daughter strand receives one-half of the amount of CENP-A originally present in the original DNA strand. To maintain the integrity of the centromere this must be replenished in G1 phase of the following cell cycle. Intriguingly, hsCYK-4 interacts with the CENP-A licensing protein hsKNL2 and localizes to centromeres in G1, and siRNA mediated knockdown of hsCYK-4 causes a loss of newly synthesized CENP-A from cells. Importantly, depletion of either Ect2 or Cdc42 phenocopies the effect of hsCYK-4 knockdown on CENP-A content. As none of these proteins affect the rate of synthesis of CENP-A, they must be important for maintenance of newly synthesized CENP-A within the centrosome [45]. Thus, this finding may represent a mechanistic basis explaining how Ect2 and Cdc42 control spindle attachments to the kinetochore [97,98], as kinetochore integrity would be severely compromised by loss of CENP-A.

In addition to Ect2, CYK-4 controls the recruitment of other proteins during cytokinesis. For example, the cytoskeleton interacting protein anillin is recruited to the equatorial membrane and cleavage furrow by interaction with CYK-4. This is an essential event, as deletion of anillin inhibits cytokinesis [82,83]. Anillin recruitment has been demonstrated most effectively in *Drosophila*, where localization of anillin to the cortical cleavage site occurs by interaction with a small population RacGAP50 (CYK-4) associated with peripheral microtubules. Interaction of anillin with the cleavage site is then reinforced by binding to cortical F-actin and myosin [23,30]. Another important interaction is the recruitment of FIP3 to the midbody during late cytokinesis. FIP3 is a Rab11 and ARF6 interacting protein that was originally shown to be important for endosome recycling. However, it was later found to also be essential for cytokinesis [92]. In this role FIP3 is needed for the delivery of membranes to the cleavage furrow and site of abscission. Interaction of hsCYK-4 with FIP3 occurs through a region of FIP3 distinct from its Rab11/ARF6 binding site, and both hsCYK-4 and FIP3 co-localize at the midbody. The binding of Ect2 and FIP3 to hsCYK-4 is mutually exclusive; however, Ect2 does not compete with hsCYK-4 for FIP3 recruitment to the midbody. Importantly, recruitment of FIP3 by hsCYK-4 is essential for abscission to occur [80].

4.2. Other RhoGAPs in mitosis and cytokinesis

p190-RhoGAP isoforms play major roles in controlling RhoA inactivation kinetics during cell adhesion and migration [46], and they have also been implicated in controlling mitosis and cytokinesis. As described above, RhoA activation promotes an increase in cortical rigidity as cells enter mitosis, and early studies indicated that this is accompanied by a decrease in the GAP activity of endogenous p190-RhoGAP [52]. Soon thereafter p190A-RhoGAP was shown to localize to the cleavage furrow during cytokinesis. Accordingly, overexpression of p190A-RhoGAP caused abnormal positioning of the cleavage furrow and resulted in the accumulation of multinucleated cells [85]. These data suggested that p190A-RhoGAP and Ect2 play opposing roles in controlling RhoA activation during cytokinesis, and in fact overexpression of Ect2 can rescue cells from cytokinesis defects arising from p190A-RhoGAP overexpression [59,86]. Cells downregulate p190A-RhoGAP expression during metaphase through ubiquitylation and proteasome-mediated degradation [85]. p190A-RhoGAP is ubiquitylated at four lysines within its N-terminal GTP binding domain, an event which is necessary for cytokinesis, and expression of a mutant form of p190A-RhoGAP lacking these ubiquitylation sites is especially potent at causing cytokinesis failure [53]. Taken together these data indicate that cells must downregulate p190A-RhoGAP in order for cytokinesis to occur.

Recent data indicates that p190B-RhoGAP contributes to earlier stages of mitosis. Endogenous p190B-RhoGAP localizes to centrosomes in MCF7 cells and is tyrosine phosphorylated in pro-metaphase. Tyrosine phosphorylation of p190-RhoGAP has been shown previously to stimulate its GAP activity [3]. Importantly, p190B-RhoGAP knockdown causes defects in chromosome alignment during metaphase and chromosome segregation during anaphase, and this can be rescued by treatment with the Rac1 inhibitor NSC-23766 [36]. These data suggest that p190B-RhoGAP normally downregulates Rac1 activity in MCF7 cells to promote proper spindle assembly, and are consistent with the potent Rac1 GAP activity initially observed for p190-RhoGAP proteins *in vitro* [16,79].

Current work indicates that additional RhoGAPs contribute to cytokinesis. For example, p50-RhoGAP (ARHGAP1) localizes to the midbody during cytokinesis and is required for efficient cell division. It is delivered to the midbody by FIP3-positive endosomes and is specifically required for disassembly of cortical F-actin present in the intracellular bridge prior to abscission [77]. A previously uncharacterized RhoGAP, M phase GAP (MP-GAP) has recently been shown to limit RhoA activation in the cleavage zone during cytokinesis [103]. This was discovered through a siRNA screen for RhoGAPs that were required for cytokinesis in *C. elegans*, which identified CYK-4 and two other RhoGAPs, RGA-3 and RGA-4. As RGA-3 and RGA-4 are not conserved in the human genome, the authors performed a second, directed siRNA screen to identify RhoGAPs that suppress mitotic membrane blebbing associated with excessive RhoA activation. This screen identified only one RhoGAP, MP-GAP (ARHGAP11A), whose knockdown resulted in mitotic membrane blebbing. MP-GAP localizes to the plasma membrane in early mitosis and to the equatorial membrane in anaphase. Knockdown of MP-GAP caused cytokinesis failure in 17% of HeLa cells, which could be rescued by treatment with C3 toxin, the ROCK inhibitor Y-27632, or an inhibitor of Plk1. Interestingly, MP-GAP knockdown increased the rate of RhoA activation as cells entered anaphase, but did not alter the localization of RhoA or anillin within the equatorial plasma membrane. Thus, MP-GAP, rather than CYK-4, performs the important role of limiting the duration of RhoA signaling during cytokinesis.

5. RhoGDIs in mitosis and cytokinesis

There are three RhoGDIs in the human genome, each of which binds to inactive, GDP-bound Rho proteins to sequester them in the cytoplasm and in some cases protect them from degradation [15,25]. However, only two studies have implicated RhoGDIs in mitotic regulation. In the first, microinjection of recombinant RhoGDIα into *Xenopus* embryos

prevented cleavage furrow formation and cytokinesis [43]. Since this also prevented accumulation of F-actin in the in the cleavage furrow, RhoGDIα presumably functioned in this context by sequestering RhoA from RhoGEFs such as Ect2. In the second study, endogenous RhoGDIβ was found to be overexpressed in many cancer cell lines and to localize to centrosomes. In addition, overexpression of RhoGDIβ slowed progression through mitosis in HeLa cells, and siRNA mediated knockdown of RhoGDIβ resulted in cells with monopolar spindles. This significantly inhibited cytokinesis, as there was an increase in cells with 4 N and 8 N DNA content [39].

6. Conclusions

Although their specific functions vary among cell types, it has become abundantly clear that Rho GTPase function is essential for mitosis and/or cytokinesis in all animal cells. Moreover, because of the inherently inefficient GTPase cycle of Rho proteins, RhoGEF and RhoGAP activities are indispensable to their regulation. In this regard, much remains to be learned of the roles and regulation of particular Rho regulatory proteins in mitosis and cytokinesis. For example, the roles of p190-RhoGAP, p50-RhoGAP and MP-GAP in cytokinesis are only now being uncovered, and it is still not clear how their activities are regulated. Similarly, the ways in which the activities of Ect2, GEF-H1 and MyoGEF are coordinated during cytokinesis are not entirely clear. The roles of particular RhoGEFs and RhoGAPs during mitosis are even less well delineated. For example, it is not well understood how microtubule–kinetochore attachments are regulated by the GEF-H1/RhoA/mDia1 and Ect2/Cdc42/mDia3 pathways. In addition, the molecular mechanisms by which the RhoGEFs ARHGEF10, Tiam1 and Net1 contribute to their particular mitotic events have only been touched upon.

Maintenance of genomic stability is fundamentally important for all cells, and aberrant mitotic function can lead to deleterious outcomes including cell death, senescence, and neoplastic transformation. Thus, altered expression or regulation of Rho regulatory proteins have the potential to lead to human diseases such as premature aging or cancer. For instance, the RhoGEFs Ect2, GEF-H1 and Net1 are all overexpressed in breast cancer, yet it is not known whether this contributes to genomic instability and tumorigenesis. In the case of Net1 it is particularly easy to envision how this may cause genomic instability, as relatively small changes in its level of expression cause profound mitotic defects [58]. An important area for future research will be to determine whether altered regulation of particular Rho regulatory proteins contributes to genomic instability and ultimately human disease.

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