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Cytokinesis and cancer: Polo loves ROCK'n' Rho(A)

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Abstract

Cytokinesis is the last step of the M (mitosis) phase, yet it is crucial for the faithful division of one cell into two. Cytokinesis failure is often associated with cancer. Cytokinesis can be morphologically divided into four steps: cleavage furrow initiation, cleavage furrow ingression, midbody formation and abscission. Molecular studies have revealed that RhoA as well as its regulators and effectors are important players to ensure a successful cytokinesis. At the same time, Polo-like kinase 1 (Plk1) is an important kinase that can target many substrates and carry out different functions during mitosis, including cytokinesis. Recent studies are beginning to unveil a closer tie between Plk1 and RhoA networks. More specifically, Plk1 phosphorylates the centralspindlin complex Cyk4 and MKLP1/CHO1, thus recruiting RhoA guanine nucleotide-exchange factor (GEF) Ect2 through its phosphopeptide-binding BRCT domains. Ect2 itself can be phosphorylated by Plk1 *in vitro*. Plk1 can also phosphorylate another GEF MyoGEF to regulate RhoA activity. Once activated, RhoA-GTP will activate downstream effectors, including ROCK1 and ROCK2. ROCK2 is among the proteins that associate with Plk1 Polo-binding domain (PBD) in a large proteomic screen, and Plk1 can phosphorylate ROCK2 *in vitro*. We review current understandings of the interplay between Plk1, RhoA proteins and other proteins (e.g., NudC, MKLP2, PRC1, CEP55) involved in cytokinesis, with particular emphasis of its clinical implications in cancer.

Keywords: Polo-like kinase 1; RhoA GTPase; Rho kinase; cytokinesis

Introduction

Cytokinesis, the last step of the M (mitosis) phase, involves physically dividing the cytoplasm of a single cell to form two daughter cells. This is a crucial step in cell cycle and has been widely studied in many model organisms: budding yeast, fission yeast, *Drosophila*, *Caenorhabditis elegans*, *Xenopus*, *Dictyostelium*, plants, and vertebrate cells (Normand and King, 2010). In animal cells the contractile ring carries out the cytokinesis step and is composed of the actin cytoskeleton and its motor molecule, myosin II (referred to as myosin in this review). But what are the regulatory proteins for the spatial and temporal events of cytokinesis? The small GTPase of Rho (Ras homologous) families are among the first proteins to be identified. Mammalian Rho GTPases comprise 20 intracellular signaling molecules, and can be subdivided into three major subsets: Rho, Rac and Cdc42 (Narumiya and Yasuda, 2006). They cycle between the inactive GDP-bound form and the active GTP-bound form. The cycling of Rho GTPases between these two states is regulated by three sets of proteins, guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine

nucleotide-dissociation inhibitors (GDIs). All three subsets of Rho GTPases are implicated in cytokinesis in different organisms, but RhoA is the most critical in mammalian cells. During cytokinesis, both induction and progression of the contractile ring depend on RhoA activation (Piekny et al., 2005).

Besides the cytoskeleton system and its interacting Rho GTPase, a successful cytokinesis also requires key protein kinases and signaling networks to coordinate the position of chromosomes in relative of the cell cortex. Cyclin-dependent kinases (Cdks), Aurora B and Polo-like kinases (Plks) are important kinases that not only regulate cytokinesis, but also are crucial regulators of other mitotic events (Glotzer, 2005; Barr and Gruneberg, 2007). There are several conserved Plks in humans, and we will only focus on Plk1 in this review, since it is believed that the major function is attributed to Plk1. Recently, some substrates of Plk1 have been identified to be involved in cytokinesis, including PRC1 (Protein Regulator of Cytokinesis 1) (Neef et al., 2007), CEP55 (CEntrosome Protein 55) (Fabbro et al., 2005), NudC (Nuclear-distribution gene C) (Zhou et al., 2003) and MKLP2 (mitotic-kinesin-like protein 2) (Neef et al., 2003). Also among these substrates are the Rho proteins: Rho GEF Ect2 (Epithelial cell transforming gene 2) (Niiya et al., 2006), Rho GAP HsCyk-4 (Burkard et al., 2009; Wolfe et al., 2009) and MKLP1/CHO1 (Liu et al., 2004). Moreover some of the RhoA downstream effectors are found to bind to the Plk1 Polo-box domain (PBD), including the Rho-associated coiled-coil-forming kinase (ROCK) (Lowery et al., 2007). ROCK is also phosphorylated by Plk1 *in vitro* (Lowery et al., 2007). Thus Plk1 and Rho GTPases are intricately linked with each other during the cytokinesis process.

It has been widely known that cytokinesis failure results in polyploidy and increased genome instability, which are frequently observed in cancer cells. In fact, Plk1, RhoA and their interacting proteins are all reported to be deregulated in some cancers. As more and more proteins involved in tumorigenesis are found to play a role in cytokinesis, such as Chk1 (Peddibhotla et al., 2009) and BRCA2 (Daniels et al., 2004), it has become apparent that cytokinesis and cancer are interconnected. This review will focus on these recent new findings in vertebrate cells and will explore its potential implication in cancer therapy, but observations from yeast and other organisms are discussed where appropriate.

The structure of Plk1 and its function in cytokinesis

Plk1 is a serine/threonine kinase that orchestrates the mitotic process. It was first discovered in *Drosophila,* as *polo* mutants fail to undergo a normal mitosis (Sunkel and Glover, 1988). And Plk1 homologues have been identified in many eukaryotes (Table 1). Plk1 has been shown to play key roles during different stages of mitosis, including mitotic entry, bipolar spindle formation, chromosome segregation and cytokinesis (Barr et al., 2004; van de Weerdt and Medema, 2006).

The structure of Plk1 is conserved across different species, with a serine/threonine kinase domain at its N-terminus and a regulatory domain, the PBD, at its C-terminus (Fig. 1A). Plk1 is activated by phosphorylation at Thr210 within the kinase domain. All Plks have a conserved PBD, and PBD has been identified as a phosphopeptide-binding motif (Elia et al., 2003). Indeed, studies

Table 1
Homologues of relevant proteins in eukaryotes

|	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Drosophila melanogaster*	*Caenorhabditis elegans*	Mammals
Polo-like kinase 1	Cdc5	Plo1	Polo	Plc1	Plk1
Rho A	Rho1	Rho1	Rho	Rho A	Rho A
ROCK	NA	NA	Rok/Drok	LET-502	ROCK
RhoGEF/Ect2	Tom2, Tus1	NA	Pebble(Pbl)	Let-21	Ect2
GAP	NA	NA	Tumbleweed/MgcGAP50C	Cyk-4	MgcRacGAP/HsCyk-4
MKLP1	NA	NA	Pavarotti	ZEN-4	MKLP1/CHO1/Kif23
MYPT1	NA	NA	MYPT/Mbs	MEL-11	MYPT1

NA: no homologs available.
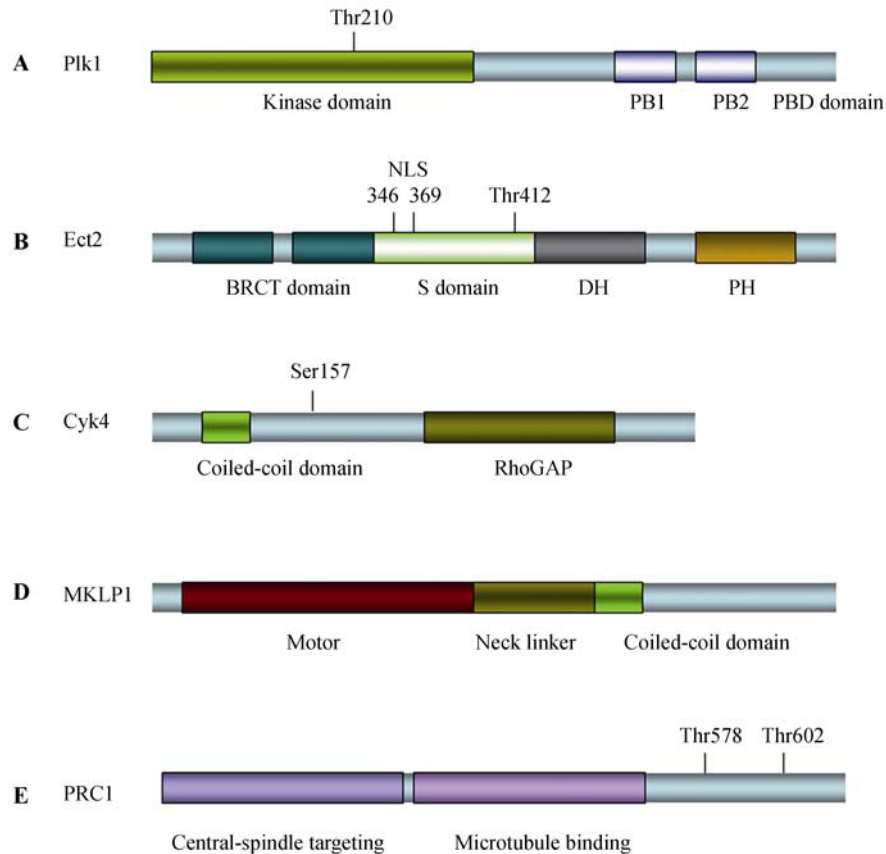
Fig. 1. Diagrams of protein structures. **A:** Plk1 consists of a serine/threonine kinase domain at its N-terminus and a regulatory domain and the polo box domain (PBD) at its C-terminus. **B:** Ect2 consists of two phosphopeptide-binding BRCT domains at its N-terminus and a tandem array of Dbl-homology (DH) domain and pleckstrin-homology (PH) domain at its C-terminus **C:** Cyk4 consists of an N-terminal coiled-coil domain and a C-terminal RhoGAP domain. **D:** MKLP1 consists of an N-terminal motor domain and a short coiled-coil region. **E:** PRC1 contains a central-spindle targeting at its N-terminus, and a central microtubule binding domain.

of Plk1 and its substrates have established a common theme that Plk1 can dock to specific phosphorylated targets through its PBD domain.

Early evidence showing Plk1's function in cytokinesis comes from *S. pombe* Plo1 (Ohkura et al., 1995). Plo1 activity correlates with division septum formation, as up- or down-regulation of Plo1 both affects the division septum. The study of the role of Plk1 in mammalian cell cytokinesis is hampered by the fact that Plk1 depletion causes early mitotic defects. But overproduction of Plk1 results in multinucleation in mammalian cells, indicative of cytokinesis failure. Plk1's substrates during cytokinesis include MKLP2 and NudC. Both MKLP2 and NudC have motor protein activity (MKLP2 is a kinesin and NudC is a component of the dynein), and both localize to the central spindle. Plk1 phosphorylates MKLP2 at Ser528 and phosphorylated MKLP2 binds with Plk1 PBD (Neef et al.,

2003). When this phosphorylation is blocked, cells show cytokinesis defects. *NudC* RNA interference (RNAi) results in multinucleation and midbody arrest (Zhou et al., 2003). NudC is phosphorylated by Plk1 at Ser274 and Ser326 *in vitro*, and phosphorylation-deficient mutants will not rescue the cytokinesis defects of *NudC* RNAi.

Direct evidence of Plk1's involvement in RhoA mediated cytokinesis pathway comes from chemical studies (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007; Santamaria et al., 2007). Burkard et al. (2007) disrupted Plk1 and substituted it with a mutant Plk1[as]. Plk1[as] has an enlarged catalytic pocket that can accommodate bulky purine analogs (e.g., 1-NM-PP1, or 3-MB-PP1). Since these analogs will not fit into the wild-type Plk1, they can specifically block the activity of Plk1[as]. When the purine analogs are applied during anaphase, Plk1's recruitment to the central spindle is blocked, which prevents

cleavage furrow formation and cell division. Closer examination reveals that RhoA localization to the cleavage cortex is disrupted, and so are the RhoA downstream targets including citron kinase and anillin. Ect2's recruitment to the equatorial cortex and central spindle is also affected. Another method directly uses Plk1's small-molecule inhibitor BI 2536 (Petronczki et al., 2007). BI2536 treatment abolishes Ect2 localization to the central spindle, but Cyk4 and Mklp1 are not affected. Other inhibitors such as BTO-1 and ZK-Thiazolidinone (TAL) have the same effect (Brennan et al., 2007; Santamaria et al., 2007). These studies reveal that Plk1 plays important roles during the last stages of cell cycle.

Plk1 and RhoA: caught in the act of cytokinesis

There are four morphological stages of cytokinesis: division site positioning and cleavage furrow initiation; cleavage furrow ingression; midbody formation; and abscission (Fig. 2). We are going to review the roles of Plk1, RhoA and their relationship at each distinct stage. Table 2 summarizes the currently known Plk1 substrates and interacting proteins involved in cytokinesis.
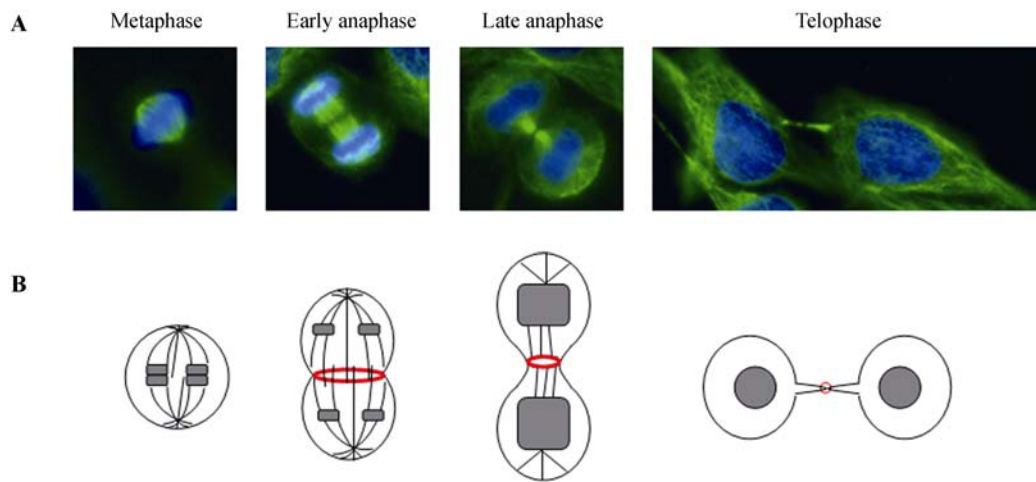

Fig. 2. The mitotic cell cycle. **A:** HeLa cells in different phases of mitosis were stained with anti-tubulin (green) antibodies and DAPI. **B:** Schematic diagrams of the spindle assembly in relative to the chromosomes during mitosis. Red circle demarcates the contractile ring.

Table 2
Some of the known Plk1 substrates and interacting proteins in cytokinesis and their phosphorylation sites

Protein	Phosphorylation site	Biological function of phosphorylation	Reference
Rock2	Multiple sites	Binds with Plk1 PBD, activates ROCK2 activity	Lowery et al., 2007
MgcRacGAP/HsCyk-4	Ser157	Binds to the BRCT domain of Ect2, thus recruiting Ect2 to the midzone	Burkard et al., 2009; Wolfe et al., 2009
CHO1/MKLP1	Ser904, Ser905	Binds with Plk1 PBD, essential for cytokinesis	Liu et al., 2004
MyoGEF	Thr574	Regulates RhoA activity	Asiedu et al., 2008
MKLP2	Ser528	Binds with Plk1 PBD, essential for cytokinesis	Neef et al., 2003
NudC	Ser274,Ser26	Essential for cytokinesis	Zhou et al., 2003
Cep55	Ser436	Essential for cytokinesis	Fabbro et al., 2005
Ect2	Thr412 (by Cdk1)	Binds with Plk1 PBD, regulates RhoA recruitment and activation	Niiya et al., 2006
PRC1	Thr578, Thr602	Binds with Plk1 PBD in an anaphase-specific manner, essential for cytokinesis	Neef et al., 2007

Division site positioning and cleavage furrow initiation

In different organisms, division site positioning is established at different points in the cell cycle. Cytokinesis initiation in various systems was reviewed by Oliferenko et al. (2009). In animal cells the site of cell division is chosen during mitosis. More specifically, it is determined in late anaphase and/or telophase. During this stage, the mitotic spindle pulls the sister chromatids apart, and in the mean time emits signals to initiate cytokinesis. Earlier micromanipulation experiments show that when mitotic spindle orientation in star fish eggs is changed, the position of the cortical contractile ring is also altered (Rappaport and Ebstein, 1965), indicating that the spindle position specifies the cleavage furrow formation. The mitotic spindle consists of the interdigital central spindle and polar astral microtubules. Central spindle may send positive signals to the cell cortex nearest to the spindle midzone to specify cleavage sites, while the polar astral spindle may inhibit the cortical contractility. These distinct groups of spindles cooperate together to signal the furrowing site (Bringmann and Hyman, 2005; Glotzer, 2009).

How can the spindle microtubule control the cortical contractility? The answer lies in the small GTPase RhoA. RhoA is crucial for furrowing, as biochemical inactivation or depletion of RhoA will lead to cleavage furrow formation failure (Piekny et al., 2005). Activated RhoA localizes to a narrow cortical zone within the cleavage furrow, and spindle displacement can perturb this localization pattern (Bement et al., 2005). Activated GTP-bound RhoA in turn induces F-actin assembly and activates myosin function, thus promoting the contractility of cell cortex. The specificity of RhoA activation is achieved by localizing specific RhoA regulators on the microtubules. The regulators that have been identified so far include Ect2, Cyk4, MyoGEF (<u>myo</u>sin-interacting <u>GEF</u>), p0071, and phospholipids (details will be discussed below).

Ect2 was originally isolated as a proto-oncogene from epithelial cells that are capable to transform (Miki et al., 1993). The C-terminus of Ect2 contains a tandem array of Dbl-homology (DH) domain and pleckstrin-homology (PH) domain (Fig. 1B). It is the C-terminus that confers Ect2 the ability to catalyze guanine nucleotide exchange on RhoA (Saito et al., 2004). Besides the DH/PH cassette, Ect2 contains N-terminal tandem BRCT (<u>BR</u>CA1-<u>C</u> Terminal) domains, and the central S domain that contains the nuclear localization signals. The BRCT domains associate with the C-terminal DH/PH domain and blocks its ability of guanine nucleotide exchange (Saito et al., 2004). Ect2 localizes in the nucleus during interphase in an inactive state, becomes activated and localizes to the mitotic spindles during metaphase, and finally appears at the midbody structure during cytokinesis. Both Ect2 antibody injection and *Ect2* RNAi inhibits cytokinesis and leads to multinucleated cells (Saito et al., 2004). These results demonstrate that Ect2 plays a crucial role to activate RhoA.

How is Ect2 recruited to the central spindle? It turns out that the centralspindlin complex recruits Ect2. The tetrameric centralspindlin complex is composed of a dimer of the kinesin 6 protein MKLP1 (also known as Kif23) and a dimer of the GAP Cyk4 (also known as RacGAP1 or MgcRacGAP) (Mishima et al., 2002). The interaction between MKLP1 and Cyk4 is evolutionarily conserved, and they localize to the central spindle in an inter-dependent manner, where they promote the microtubule bundling (Mishima et al., 2002). The N-terminus of Cyk4 binds to the neck linker of MKLP1 (Fig. 1, C and D), and assembles into a stable centralspindlin complex (Pavicic-Kaltenbrunner et al., 2007). Cyk4 binds to and stabilizes activated Ect2, allowing Ect2 to interact with RhoA (Yuce et al., 2005). As a result, Ect2 activates RhoA and signals to the overlying equatorial cortex, leading to contractile ring formation and cleavage furrow ingression. The centralspindlin complex travels along central spindles as higher-order clusters and accumulates at the midbody. Centralspindlin clustering is critical for microtubule bundling and motility, as well as midbody formation (Hutterer et al., 2009). When the centralspindlin complex localizes to the central spindle, it restricts Ect2 within the narrow zone, leading to RhoA's narrow activation. When this localization pattern is disrupted, RhoA will be activated in a much broader range, and cells will fail to form a furrow. In *Xenopus*, MgcRacGAP not only anchors active RhoA to the activity zone, but also promotes local RhoA inactivation that provides constant Rho to the GTPase cycle (Miller and Bement, 2009), consistent with earlier findings that the GAP activity is essential for cytokinesis in mammals (Hirose et al., 2001).

It may seem paradoxical that both Rho GEF and GAP are required for RhoA activation. When *MgcRacGAP* antisense morpholino oligonucleotides are applied, the *Xenopus* embryos display cytokinesis defects as well as a broader zone of RhoA activity (Miller and Bement, 2009). Only wild-type *MgcRacGAP*, but not a GAP activity mu-

tant allele of *MgcRacGAP* can rescue the defects, suggesting that the GAP activity is required for RhoA inhibition, which in turn is required for cytokinesis. Thus the "Rho flux model" speculates that a weaker GAP activity than the GEF activity at the cleavage furrow will lead to RhoA activation, but both the GAP and GEF are required. The other "Rac inactivation" model states that RacGAP is required to inhibit Rac GTPase and activate RhoA at the same time (Canman et al., 2008). Further experiments may reconcile these two different models, but there is no doubt that GAP is essential for RhoA activity.

Recent investigations show that both GEF Ect2 and Cyk4 are Plk1 substrates. Ect2 is phosphorylated during the G2/M transition (Niiya et al., 2006). *In vitro* Ect2 can be phosphorylated by Cdk1 and Plk1. Cdk1 phosphorylation at Thr412 creates a phosphospecific binding pocket (SpTP) for Plk1 PBD (Niiya et al., 2006). When the Thr412 residue is mutated to Ala, RhoA activation is diminished. In contrast, the phosphomimic Ect2 T412D mutant still exhibits significant action of RhoA. Ect2 overproduction induces cortical hyperactivity that leads to cell death, but Ect2-T412A overproduction has no such effect (Niiya et al., 2006). Furthermore, this interaction is not limited to mammalian cells. In budding yeasts two RhoA GEFs (Tus1 and Rom2) are both substrates of Cdc5 *in vitro* and *in vivo* (Yoshida et al., 2006). Thus it seems a conserved pathway for Plk1 to regulate the recruitment and activation of RhoA by regulating Rho GEF's localization to the division site.

Since the interaction between Ect2's BRCT domains and Cyk4 is dependent on phosphorylation (Yuce et al., 2005), there has been speculation that Cyk4 might be phosphorylated prior to binding to Ect2's BRCT domains, which bind phosphopeptides. Indeed, two independent investigations show that Plk1 specifically binds and phosphorylates Cyk4 at Ser157, thus creating a docking site for the Ect2 BRCT domains (Burkard et al., 2009; Wolfe et al., 2009). When Ser157 is mutated to a nonphosphorylated form, Ect2 fails to localize at the midzone, leading to cleavage furrowing errors (Burkard et al., 2009; Wolfe et al., 2009).

Besides Ect2, RhoA also has other regulators, including GEF MyoGEF (Wu et al., 2006), the armadillo protein p0071 (Wolf et al., 2006) and phospholipids (Yoshida et al., 2009). MyoGEF also contains the DH and PH domains. *MyoGEF* disruption by RNAi results in binucleated and multinucleated cells and decreased RhoA activation (Wu et al., 2006). MyoGEF localizes to the central spindle, and interacts with Ect2, and *MyoGEF* RNAi leads to Ect2 and RhoA mislocalization during cytokinesis (Asiedu et al., 2009). MyoGEF and Plk1 colocalize at the spindle pole and central spindle, and MyoGEF localization is dependent on Plk1 (Asiedu et al., 2008). Plk1 phosphorylates MyoGEF on Thr574 *in vitro* (Asiedu et al., 2008). *In vivo* experiments show that the MyoGEF T574A mutant dramatically decreases MyoGEF phosphorylation. MyoGEF T574A displays decreased GEF activity towards RhoA, suggesting that Plk1 can regulate RhoA activity through phosphorylating MyoGEF.

The armadillo protein p0071 localizes at the midbody (Wolf et al., 2006). Its upregulation or downregulation leads to cytokinesis defects and induces apoptosis. It turns out that p0071 interacts with Ect2 and RhoA, and alteration of p0071 expression deregulates RhoA activity (Wolf et al., 2006). p0071 localization is dependent on kinesin-II member Kif3b (Keil et al., 2009).

Evidence that phospholipids promote RhoA localization comes from the budding yeast. During septation and abscission in the yeast cells, Rho1 bud neck targeting requires that the Rho1 polybasic sequence binds to acidic phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP2) (Yoshida et al., 2009). In animal cells, this mechanism might be further facilitated by phosphatidylinositol 3,4,5-trisphosphate, which is not present in the budding yeast.

Cleavage furrow ingression

After RhoA is temporally and locally activated, it will recruit and activate downstream effectors to induce cleavage furrow ingression (Fig. 3). Downstream targets include proteins to stimulate actin polymerization, as well as ROCK and citron kinase that stimulate myosin activity (Matsumura, 2005).

Myosin comprises two heavy chains, two essential light chains and two regulatory myosin light chains (MLC). MLC Ser19 phosphorylation stimulates myosin ATPase activity, and Thr18 phosphorylation promotes myosin assembly (Matsumura, 2005). During mitosis, MLC is phosphorylated at Ser1, 2 and 9 by CDK1 to inhibit myosin ATPase activity. CDK1 deactivation at anaphase allows MLC dephosphorylation at these sites. Thus specific MLC phosphorylation site controls contractile ring assembly. Expression of a nonphosphorylation mutant of MLC in fly and culture cells both results in cytokinesis failure (Jordan and Karess, 1997; Komatsu et al., 2000).
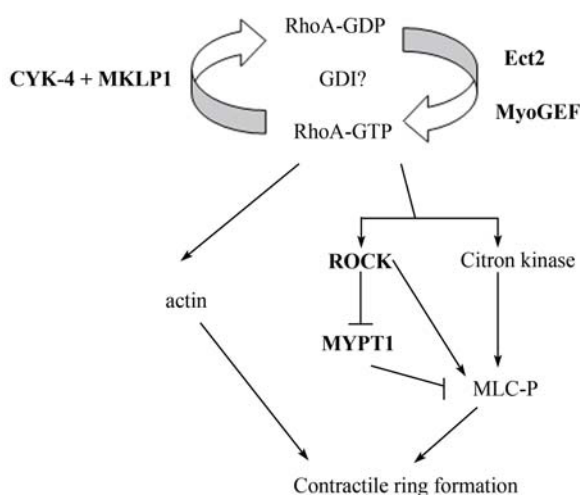
Fig. 3. Regulation of the contractile ring formation by the RhoA GTPase. RhoA is activated by GEF (Ect2, MyoGEF) and inactivated by GAP (Cyk4). RhoA-GTP is concentrated at the cleavage site and induces actin filament assembly. In the meantime, RhoA activates downstream effectors (ROCK and citron kinase), which induce MCL phosphorylation and thereby myosin II filament formation. Thus the contractile ring, which is composed of the actin filaments and myosin II, is assembled. In this diagram, proteins marked in bold are currently known Plk1 substrates.

MLC phosphorylation is controlled by three kinases: ROCK, citron kinase, MLCK (Myosin Light Chain Kinase), and also reversibly controlled by myosin phosphatase. ROCK localizes to the cleavage furrow, and inhibition of ROCK with Y-27632 causes cleavage delay, but not affecting cytokinesis initiation and completion (Kosako et al., 2000). Mammalian cells have two ROCK isoforms: ROCK1 (Rokβ) and ROCK2 (Rokα). Knockdown of either *ROCK1* or *ROCK2* in mice does not display cytokinesis defects (Thumkeo et al., 2003; Shimizu et al., 2005), which might be caused by the redundancy between these two kinases. Double-knockout mice will help to elucidate the function of ROCK in cytokinesis.

Both cytology and biochemistry studies show that Plk1 and ROCK2 interact. Plk1 and ROCK2 colocalize at the midbody during cytokinesis (Lowery et al., 2007). Plk1 coimmunoprecipitates with ROCK2 in a phosphorylation-dependent and mitosis-specific manner. Plk1 can phosphorylate ROCK2 *in vitro*, and phosphorylated ROCK2 interacts with Plk1 PBD (Lowery et al., 2007). Plk1 and RhoA act in a synergistic fashion to activate ROCK2 *in vitro* and *in vivo*.

The second kinase, citron kinase, also localizes in the cleavage furrow. Its overproduction upregulates cortex

contractility, suggesting that it positively regulates myosin activity (Madaule et al., 1998). It phosphorylates MLC at Ser19/Thr18 both *in vitro* and *in vivo* (Yamashiro et al., 2003). Citron kinase knockout mice complete embryonic development, but some neuronal precursor cells display abnormal cytokinesis and massive apoptosis (Di Cunto et al., 2000). These cells express ROCK proteins at the normal level, suggesting that although ROCK and citron kinase might be redundant in activating myosin, they may have other distinct regulatory function.

MLCK is a third kinase that can phosphorylate MLC in the cleavage furrow. It is activated by Ca^{2+}/calmodulin and also by phosphorylation. MLCK inhibition in cultured mammalian cells leads to cytokinesis failure (Normand and King, 2010). MLCK might also be regulated by phospholipids, because PIP2 hydrolysis is important for inositol-1,4,5-trisphosphate (IP3)-induced calcium release (Wong et al., 2007).

The only known phosphatase of MLC is myosin phosphatase, which consists of a targeting subunit MYPT1 (Myosin Phosphatase Targeting Subunit 1), a catalytic subunit PP1Cβ and an additional small subunit (Baumann et al., 2007; Matsumura and Hartshorne, 2008). During anaphase, both ROCK and Aurora B kinase phosphorylate MYPT1 in the furrow to inhibit its phosphatase activity (Kawano et al., 1999; Yokoyama et al., 2005), which may lead to increase of MLC phosphorylation at the cleavage furrow and signal for cytokinesis. MYPT1 is recently reported to be phosphorylated by Cdk1 in a mitosis-specific fashion, which generates a binding motif for Plk1 PBD (Yamashiro et al., 2008). MYPT1 antagonizes Plk1 activity, as MYPT1 depletion increases Plk1-T210 phosphorylation (Yamashiro et al., 2008). But whether this interaction functions in cytokinesis remains to be elucidated. MYPT1 is recently found to be a regulatory subunit of serine/threonine-protein phosphatase 6 catalytic subunit (PP6C) and function in the homologous recombination pathway (unpublished data). It is a possible scenario that PP6C is targeted by MYPT1 to dephosphorylate MLC.

Formation of the midbody

Cleavage furrow ingression continues until the actomyosin contractile ring comes into close proximity to the central spindle. Although proteomic approaches have been used to identify the midbody components (Skop et al., 2004; Chen et al., 2009a), its precise function is not yet

understood in detail. It is hypothesized that the midbody maintains a state of constriction until abscission is completed.

The centralspindlin complex also plays an essential role in this step. MKLP1 has a splice variant called CHO1. CHO1 is shown to be required for midbody formation in mammalian cells in RNAi studies (Matuliene and Kuriyama, 2002, 2004). CHO1 contains an extra F-actin interacting domain, indicating that it may function to link the actin with the centralspindlin complex. Plk1 interacts with CHO1 during anaphase and telophase, and the PBD domain of Plk1 is responsible for this association (Liu et al., 2004). Also needed is the stalk domain of CHO1. When CHO1 is depleted, Plk1 fails to localize at the midbody, and cells turn multinucleated with more centrosomes. Plk1 can phosphorylate CHO1 *in vitro*, and Ser904 and Ser905 are the major phosphorylation sites. When a non-phosphorylated form of CHO1 is expressed, cells display cytokinesis defects (Liu et al., 2004).

Some proteins that localize to the midbody have been studied in detail, and one of them is PRC1. It is critical for midbody formation in mammalian cells. In anaphase, PRC1 localizes to the central spindle where its main function is to bundle microtubules (Mollinari et al., 2002). Depletion of PRC1 leads to abscission failure without affecting the cleavage furrow ingression. Domain analysis reveals that PRC1 has two separate domains that target PRC1 to distinct subcellular structures (Mollinari et al., 2002) (Fig. 1E). Its N-terminus interacts with kinesin Kif4 and is targeted to the midzone, where PRC1 recruits the centralspindlin complex and other kinesin proteins (Zhu and Jiang, 2005). On the contrary, the central region of PRC1 is required for microtubule binding and bundling activity (Mollinari et al., 2002). In metaphase CDK1 phosphorylates PRC1 at T470 and T481 (Neef et al., 2007). These phosphorylation events prevent Plk1 binding. As CDK1 activity decreases in anaphase, Plk1 phosphorylates PRC1 at T578 and T602, creating its own docking site. Thus PRC1 binds to Plk1 in an anaphase-specific manner, which is essential for cytokinesis. Consistent with this, CDK1 phosphorylation-deficient mutations result in premature binding with Plk1 and mitotic block (Neef et al., 2007).

Plk1 also regulates the katanin protein that severs microtubules at the midbody and functions both in mitosis and meiosis (McNally et al., 2002). In *Xenopus*, Plx1 colocalizes with katanin at spindle poles *in vivo* and puri-

fied Plx1 increases the microtubule-severing activity of katanin *in vitro* (McNally et al., 2002). Katanin is also essential for post-mitotic differentiation events in vertebrate neurons and in *Arabidopsis*.

Abscission

Abscission is the final step of cytokinesis. During this stage, the microtubule bundles start to compact and disappear (Fig. 2). The centrosome protein Cep55, which localizes to the midbody in a centralspindlin-dependent manner, is essential for this step (Zhao et al., 2006). The absence of Cep55 will lead to midbody formation defects (Zhao et al., 2006). Cdk1/Erk2 phosphorylates Cep55 at Ser425/Ser428, which is required for Cep55's interaction with Plk1. Plk1 further phosphorylates Cep55 at Ser436. When Ser436 is mutated, cells display cytokinesis failure (Fabbro et al., 2005). Cep55 also has microtubule-bundling function that is essential for the midbody formation (Zhao et al., 2006). Recently, it was shown that the peptidyl-prolyl isomerase Pin1 enhances the Plk1-dependent phosphorylation of Cep55 (van der Horst and Khanna, 2009). Moreover, Cep55 is stabilized post-translationally during mitosis in a Pin1-dependent manner, and Cep55's stable protein level is essential for proper execution of cytokinesis (van der Horst et al., 2009).

Because abscission involves the membrane organization changes, the membrane trafficking system is crucial for this step. These include the secretory pathway, the endocytic pathway and the ESCRT machinery (Endosomal Sorting Complex Required for Transport) (Normand and King, 2010). Depletion of components of these pathways has been shown to lead to cytokinesis defects. Alix and Tsg101 (Tumor-Susceptibility Gene 101) are components of the ESCRT network, and they are recruited to the midbody by interacting with Cep55 (Carlton and Martin-Serrano, 2007).

Implication of cytokinesis in cancer

It has been widely known that cytokinesis failure will lead to polyploidy. But some body tissues are polyploid by nature. For instance, megakaryocyte (MK) cells are unique among mammalian cells in that they are the naturally polyploid hematopoietic cells that can give rise to platelets. Polyploidization is intrinsic to this differentiation event.

MK cell polyploidization occurs by endomitosis, which was recently shown to be caused by late cytokinesis failure related to defects in Rho/Rock signaling (Lordier et al., 2008). Thus in certain tissues Rho and ROCK can be the targets of physiological regulation.

Apart from the naturally polyploid cells, failure of cytokinesis in normal cells can cause cell death or lead to genome amplification, which is characteristic of many cancers. When diploid and tetraploid cultures are isolated from *p53*-null cells, only the tetraploid cells are transformed *in vitro* after carcinogen exposure (Fujiwara et al., 2005). In addition, only the tetraploid cells will give rise to malignant mammary epithelial cancers after transplanted into nude mice. These results indicate that tetraploidy, which is frequently the product of cytokinesis failure, can promote tumor development in *p53*-null cells. Further studies show that the *p53*-deficient tetraploid cells display aneuploidy, genomic rearrangements and amplification (Fujiwara et al., 2005). Thus, cytokinesis failure, polyploidization and erroneous chromosome segregation may form a positive feedback loop to promote tumorigenesis.

Indeed, a lot of DNA damage checkpoint proteins are recently shown to be involved in cytokinesis. These proteins have been shown to be implicated in cancer. For instance, Chk1 is a critical component of the DNA damage checkpoint network that functions in DNA replication, intra-S phase and the G2/M phase transition. Chk1$^{+/-}$ mice display Aurora B mislocalization. Moreover, Chk1 abrogation leads to cytokinesis regression and binucleation (Peddibhotla et al., 2009). Another example is BRCA2, which is a breast cancer susceptibility protein. People carrying germ-line mutations inactivating BRCA2 are predisposed to breast cancer. RNAi experiments in HeLa cells to knock down *BRCA2* lead to cytokinesis defects (Daniels et al., 2004). Furthermore, cytokinesis failure is also observed when BCCIP1, a BRCA2-interacting protein, is downregulated (Meng et al., 2007).

More proteins are found to be at the intersection of cytokinesis and tumorigenesis. The peptidyl-prolyl isomerase Pin1 that was mentioned above localizes to the midbody ring and regulates the final stages of cytokinesis by binding to Cep55 (van der Horst and Khanna, 2009). In *Pin1* knockout mice, embryonic fibroblasts show a cytokinesis delay, and depletion of Pin1 from HeLa cells also causes cytokinesis defects. Pin1 is also deregulated in many tumors, including breast, prostate and lung cancer (Bao et al., 2004). Overexpression of Pin1 promotes tumor growth,

while inhibition of Pin1 causes tumor cell apoptosis. Therefore, many Pin1 inhibitors have been developed and could be used as a novel type of anticancer drug by blocking cell cycle progression (Xu and Etzkorn, 2009). In fact, Cep55 itself is found to be upregulated in breast, colorectal and lung cancers, and Cep55 could act as a novel breast cancer-associated antigen (Inoda et al., 2009).

Not surprisingly, both Plk1 and Rho proteins are long known to be implicated in cancer. An example is the relationship between Plk1 and p53. Plk1 depleted cells undergo apoptosis, activate caspase 3 and form fragmented nuclei (Liu and Erikson, 2003). Plk1 is later found to physically interact with p53 (through both coimmunoprecipitation and colocalization studies) and inhibit p53's pro-apoptotic function (Ando et al., 2004). Plk1 might negatively regulate p53 through Topors, a ubiquitin and SUMO E3 ligase (Yang et al., 2009). Plk1 phosphorylates Topors on Ser718 *in vivo*, and phosphorylated Topors inhibits p53's sumoylation, while enhancing its ubiquitination (Yang et al., 2009). Another target of Plk1 to regulate p53 might be Mdm2, as Plk1 also phosphorylates Mdm2 at Ser260, thus stimulating Mdm2-mediated p53 turnover (Dias et al., 2009). Moreover, *Plk1* knockdown decreased Mdm2 protein level (Kreis et al., 2009). On the other hand, using both p53 binding-defective human papillomavirus type-16 E6, and *p53* RNAi, Incassati et al. (2006) show that p53 represses Plk1 expression, suggesting that Plk1 is a target of p53. Thus Plk1 and p53 may form a feedback loop to function in tumorigenesis. Other members of the p53 family include p63 and p73, both of which regulate cell survival and apoptosis in tumors. Plk1 phosphorylates p63 at Ser52, which decreases p63's protein stability and suppresses apoptosis (Komatsu et al., 2009). Plk1 interacts and colocalizes with p73, and phosphorylates p73 at Thr27 (Koida et al., 2008; Soond et al., 2008). The CDK inhibitor p21$^{WAF1/CIP}$ (referred to as p21 afterwards) is induced by p73 in HeLa cells (p53-deficient), and the long-term suppression of Plk1 increases p21 protein level (Kreis et al., 2009). These data strongly suggest a relationship between Plk1 and p53 protein families.

As Plk1 plays a pivotal role in regulating mitosis, Plk1 is upregulated in many tumors, such as melanomas and lymphomas, and could be used as a prognostic marker for some cancers (Strebhardt and Ullrich, 2006). Plk1 has two functional distinct domains, thereby providing investigators two targets within the same protein to develop anti-proliferative drugs. The small molecule inhibitors that

have been developed against Plk1 have been useful in dissecting its cellular functions and might be useful in the clinic. For instance, BI 2536 is developed as an anti-cancer drug that blocks Plk1 activity with high potency *in vitro* and *in vivo* (Mross et al., 2008).

Rho GTPase functions in a broad spectrum of cell metabolism, including cytoskeleton dynamics, cell cycle progression, transcriptional regulation and cell survival, which are all important for tumorigenesis (Vega and Ridley, 2008). RhoA is shown to be involved in all stages of cancer progression. In breast cancers, in particular, RhoA is not only upregulated, but also involved in the tumor metastasis (Lin and van Golen, 2004). The RhoA/ROCK signaling pathway has been proposed to regulate actomyosin-based cortical contractility that leads to cell invasion (Vega and Ridley, 2008). Thus it is not surprising to find that RhoA protein levels are significantly increased in breast, lung and colon cancers (Gomez del Pulgar et al., 2005).

RhoA GEF *Ect2* is a proto-oncogene. *Ect2* is found to be overexpressed in glioma patients (Sano et al., 2006). In these patients, RhoA activation is broadened, and cytokinesis initiation is affected, both of which correlate with Ect2's function in RhoA regulation. The other activator, MyoGEF, is highly expressed in invasive breast cancer cell lines and infiltrating ductal carcinomas. It regulates the invasion activity of MDA-MB-231 breast cancer cells through activation of RhoA and RhoC (Wu et al., 2009). *DLC1* (Deleted in Liver Cancer 1) encodes a RhoGAP, and has been identified as a tumor suppressor gene that is deleted in cancers of the breast, colon and lung (Xue et al., 2008). Other GAPs, such as DLC2, DLC3, p190RhoGAP and GRAF are also implicated in cancer progression.

As far as RhoA effectors are concerned, so far there is no evidence showing that Citron is involved in tumor, but Citron interacting protein Kif14 is overproduced in breast and lung cancers (Normand and King, 2010). ROCK is upregulated in cancers. Moreover, ROCK not only interacts with p21 *in vivo* in Ras-transformed cells (Lee and Helfman, 2004), but also upregulates p21 in prostate cancer cells (Xiao et al., 2009). Upon stimulating a conditional active ROCK-estrogen receptor fusion protein, cyclin D1, p21, cyclin A levels are elevated, while p27[Kip1] levels are reduced (Croft and Olson, 2006). Current therapeutic strategies targeting Rho signaling in cancer have been twofold: one directly targets Rho, the other focuses on inhibiting Rho downstream effectors, such as ROCK.

The ROCK inhibitor fasudil not only is used to treat cardiovascular diseases, but also inhibits tumor progression in human and rat tumor models (Ying et al., 2006). Another ROCK inhibitor, Y-27632, can block Ras-mediated transformation of NIH3T3 cells (Sahai et al., 1999).

Outlooks and future directions

Are there more RhoA activators? Ect2, MyoGEF, p0071 are among the RhoA activators that have been identified so far. But to date over 70 RhoGEF, 60 RhoGAPs have been identified (Vega and Ridley, 2008), suggesting that there might be more RhoA activators. In light of the intricate interplay between Plk1 and RhoA, we speculate that more RhoA activators would turn out to be regulated by Plk1.

Upon cytokinesis completion, RhoA may need to be inactivated for cleavage furrow disassembly (Chalamalasetty et al., 2006). But how is RhoA inactivated? Recent evidence suggests that it may be regulated at the protein level. RhoA is shown to be ubiquitinated by Cul3-BACURD ubiquitin ligase complexes (Chen et al., 2009b). When Cul3 is lacking, RhoA degradation is affected, and actin stress fibers are aberrant. Dysfunction of the Cul3-BACURD complex attenuates the migration potential of HeLa cells and mouse embryonic fibroblasts, and affects convergent extension during gastrulation in *Xenopus* embryos (Chen et al., 2009b). However, the authors did not examine whether cells display any cytokinesis defects. Alternatively, RhoA might be inactivated by the RhoGDI. It has been known that GDP-Rho is prenylated and bound at the translation site by specific RhoGDIs until RhoGDF (RhoGDI-displacement factor) liberation, transported to its specific membrane location and activated by RhoGEF. It is conceivable that premature release of RhoA by RhoGDI will lead to RhoA deregulation. But so far only RhoGDIs in *Dictyostelium* are shown to be involved in cytokinesis (Imai et al., 2002; Rivero et al., 2002). Mammals have three isoforms of RhoGDI. Are they also involved in cytokinesis? Could they also be regulated by Plk1 phosphorylation? These questions await further studies.

It is now clear that cytokinesis and cancer are intricately linked. And targeting proteins involved in cytokinesis could become a common theme for tumor therapy. Further studies will reveal more Plk1 substrates and more RhoA regulating proteins that are involved in the cytokinesis

pathway, thus providing more targets for novel drug development that can be taken to the clinic.

Acknowledgements

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