# Chapter 6
# Cytoskeleton and Nucleotide Signaling in Glioma C6 Cells

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**Abstract** This chapter describes signaling pathways stimulated by the $P2Y_2$ nucleotide receptor ($P2Y_2R$), that regulate cellular processes dependent on actin cytoskeleton dynamics in glioma C6 cells. $P2Y_2R$ coupled with G-proteins, in response to ATP or UTP, regulates the level of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) which modulates a variety of actin binding proteins and is involved in calcium response and activates Rac1 and RhoA proteins. The RhoA/ROCK signaling pathway plays an important role in contractile force generation needed for the assembly of stress fibers, focal adhesions and for tail retraction during cell migration. Blocking of this pathway by a specific Rho-kinase inhibitor induces changes in F-actin organization and cell shape and decreases the level of phosphorylated myosin II and cofilin. In glioma C6 cells these changes are reversed after UTP stimulation of $P2Y_2R$. Signaling pathways responsible for this compensation are connected with calcium signaling. Stimulation of the Rac1 mediated pathway *via* $G_o$ proteins needs additional interaction between $\alpha_v\beta_5$ integrins and $P2Y_2Rs$. Rac1 activation is necessary for cofilin phosphorylation as well as integrin activation needed for focal complexes formation and stabilization of lamellipodium. Inhibition of positive Rac1 regulation prevents glioma C6 cells from recovery of control cell like morphology.

**Keywords** $P2Y_2$ • $PIP_2$ • Rho • Rac • Small G-proteins • Cofilin • Myosin II • Actin dynamics • Integrins • Cell migration • Glioma C6 cells

## Abbreviations

| | |
|---|---|
| DAG | Diacylglycerol |
| EGFRs | Epidermal growth factor receptors |
| FAK | Focal Adhesion Kinase |
| GEFs | Guanine nucleotide exchange factors |
| GPCRs | G protein-coupled receptors |
| $IP_3$ | Inositol-1,4,5-trisphosphate |

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| | |
|---|---|
| LIMK | LIM kinase |
| MARCKS | Myristoylated alanine rich C-kinase substrate |
| MHC | Myosin heavy chains |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| $P2Y_2R$ | $P2Y_2$ receptor |
| PAK | p21 Activated kinase |
| PDGFRs | Platelet-derived growth factor receptors |
| PI3K | Phosphatidylinositol 3-kinase |
| $PIP_2$ | Phosphatidylinositol-4,5-bisphosphate |
| PIP 5-kinase | Phosphatidylinositol-4-phosphate 5-kinase |
| PIP | Phosphoinositol phosphate |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| P-MLC | Phosphorylated myosin light chain |
| PTX | Pertussis toxin |
| ROCK | Rho-associated protein kinase |
| SSH | Slingshot homolog |
| TESK | Testis-specific kinase |

## 6.1 Introduction

The way in which simple, chemical reactions of the signaling pathways translate itself into the cell behavior is crucial for understanding of the living world. This chapter describes how the signal from one nucleotide receptor can influence and regulate complex phenomena of cell structure and motility.

This is the ability to actively change shape and travel from one location to another what we intuitively assume as one of the most fundamental attributes of being alive. Being motile is the common feature of multiple free living protista (Chung et al. 2001; Kłopocka and Pomorski 1996; Pomorski et al. 2007; Stockem and Kłopocka 1988) as well as tissue cells in higher organisms (Pomorski et al. 2004). The motility is crucial for organism development and everyday functions. While most of cells in human body can use bloodstream as a fast way of traveling long distance, the existence of blood-brain-barrier isolates cells of central nervous system from the passive way to spread around and force to use active crawling to change position. This is as true for the microglia cell looking for the inflammation as for the spreading cells of malignant glioma. The existence of the barrier between blood and brain creates very special environment, where every cell at least on some period in the life have to be actively motile.

The $P2Y_2$ nucleotide receptor is located in the very centre of signaling network regulating cell motility and cytoskeleton shape. There are two basic systems regulating cytoskeleton and motility: Rho-family pathways (Kłopocka and Rędowicz 2003, 2004; Kłopocka et al. 2005; Schmitz et al. 2000; Worthylake et al. 2001) and calcium signaling (Pletjushkina et al. 2001; Pomorski et al. 2004; Somlyo and Somlyo 2003). In this chapter we will show how $P2Y_2$ receptor signaling can influence both those systems. While the Chap. 4 focus on the calcium signaling in glia and glioma cells, here we will discuss the complex relations between this two signaling systems and show why nucleotide receptors and nucleotide signaling are so important for them.

In the chapter we will describe crucial pathways regulating glioma C6 cells cytoskeleton as well as the peculiar role of $P2Y_2$ in cross-talk between them. Finally we will show how experimental induction of this receptor may influence inhibition of classical RhoA cytoskeleton and motility-regulating pathway.

## 6.2 The Role of the P2Y$_2$ Receptor in Actin Cytoskeleton Organization

Signaling cascades evoked by the P2Y$_2$ receptor (P2Y$_2$R) stimulation operate by the sequential activation and deactivation of small G proteins, phospholipases and protein kinases that regulate cellular processes dependent on actin cytoskeleton dynamics: endo- and exocytosis, cytokinesis, intracellular transport, cell to cell adhesion and cell migration. Actin cytoskeleton reorganization in response to extracellular stimuli is crucial in these processes. The dynamic transformation of actin filaments that involves actin polymerization and depolymerization, formation and degradation of filamentous networks and bundles is regulated by integrated signaling cascades. Important factors in actin cytoskeletal reorganization are small GTPases of the Rho family, Rho, Rac, and Cdc42 (Bishop and Hall 2000; Hall 1998) and phosphoinositides, especially phosphatidylinositol-4,5-bisphosphate (PIP$_2$) that binds directly to a variety of actin regulatory proteins and modulates their functions (Sechi and Wehland 2000; Takenawa and Itoh 2001). It means that changes in PIP$_2$ levels influence the remodeling of the actin cytoskeleton. The P2Y$_2$ receptor is coupled to G$_q$, G$_o$ and G$_{12}$ proteins. G$_q$-coupled receptors stimulate phospholipase C (PLC) activity that hydrolyzes PIP$_2$ and generates two second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ mobilizes $Ca^{2+}$ from the endoplasmic reticulum, whereas DAG activates protein kinase C (PKC). Calcium transient promotes actin cytoskeleton reorganization by activation of $Ca^{2+}$-calmodulin-dependent myosin light chain kinase (MLCK). MLCK phosphorylates myosine light chains (MLCs) at Ser19 and/or Thr18 residues (Katoh et al. 2001; Somlyo and Somlyo 2003) and increases acto-myosin contractility at the cell periphery (Totsukawa et al. 2000).

The P2Y$_2$ nucleotide receptor contains the integrin-binding domain, arginine-glycine-aspartic acid (RGD), in its first extracellular loop (Lustig et al. 1996; van Rhee et al. 1998). The integrin binding domain in P2Y$_2$R enables it to interact selectively with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Erb et al. 2001) and is required for G$_o$-mediated Rac1 activation (Bagchi et al. 2005; Erb et al. 2001). Interaction with $\alpha_v\beta_5$ is also necessary for coupling the P2Y$_2$ receptor to G$_{12}$ (Liao et al. 2007).

The cross-talk between integrins and P2Y$_2$ receptors has been shown to enable receptor coupling to specific G proteins involved in cell migration. It was demonstrated that $\alpha_v$ integrin expression is required for P2Y$_2$R to induce cell migration in many cell types (Bagchi et al. 2005; Wang et al. 2005). P2Y$_2$ receptor has also been suggested to play a role in the wound-healing process (Greig et al. 2003).

In addition to modulation of ion channel activity (Chap. 3), P2Y receptors can modulate the activity of receptor tyrosine kinases. SH3-binding domains, that are necessary to bind and activate the non-receptor tyrosine kinase Src, have been identified in C-terminal tail of the P2Y$_2$. UTP stimulation causes Src activation and subsequent recruitment of the epidermal growth factor receptors (EGFRs) into a protein complex with the P2Y$_2$R that allows Src to phosphorylate EGFR (Liu et al. 2004). Responses to P2Y$_2$R stimulation depend on cell types and the cross-talk between various signaling pathways (Erb et al. 2006).

### *6.2.1 Regulation by PIP$_2$*

In response to external stimuli G protein-coupled receptors (GPCRs) and tyrosine kinase receptors (EGFRs and PDGFRs) regulate the level of PIP$_2$ by activating Rac and/or PLC. GTP-bound Rac activates phosphatidylinositol-4-phosphate 5-kinase (PIP 5-kinase) responsible for PIP$_2$ synthesis (Hartwig et al. 1995; Tolias et al. 2000), whereas activation of PLC leads to hydrolysis of PIP$_2$ and to formation of DAG and IP$_3$ (Boarder and Hourani 1998).

PIP$_2$ plays a critical role in controlling actin cytoskeleton dynamics by modulating a variety of actin regulatory proteins (Cooper and Schafer 2000). PIP$_2$ inhibits the activities of profilin, severing

proteins ADF/cofilin and capping proteins such as CapZ and gelsolin, whereas it activates the cross linking activity of α-actinin (Fukami et al. 1992). $PIP_2$ also activates ERM (ezrin/radixin/moesin) and vinculin that link actin filaments to the plasma membrane (Takenawa and Itoh 2001). WASP proteins, MARCKS and pleckstrin regulate actin cytoskeleton also in a $PIP_2$-dependent manner (Toker 2002). $PIP_2$ promotes actin nucleation and polymerization by interacting directly with profilin. Profilin sequesters actin monomers, thus preventing polymerization, and $PIP_2$ disrupts this association (Sohn et al. 1995). Actin filaments are also prevented from spontaneously polymerizing by capping proteins that cap the barbed ends. Interaction of $PIP_2$ with capping proteins dissociates them from the barbed ends of actin filaments. Uncapping of the barbed ends leads to elongation of the existing microfilaments (Takenawa and Itoh 2001; Toker 2002). The activity of cofilin is regulated by several mechanisms, but the most important are phosphorylation (Pak et al. 2008) and the binding of $PIP_2$ (van Rheenen et al. 2007). By binding unphosphorylated cofilin $PIP_2$ inhibits its actin-depolymerizing and actin-severing activity. Stimulation of $P2Y_2R$ by ATP or UTP results in activation of PLC and decrease in the level of $PIP_2$ with the subsequent release and activation of cofilin. Upon activation, cofilin diffuses from the plasma membrane to the F-actin compartment, where it binds to and severs actin filaments, which results in filaments with more free barbed ends and the formation of cofilin-G-actin complexes (van Rheenen et al. 2007). These complexes diffuse to the cytosol, where cofilin is phosphorylated and released from the cofilin-G-actin complex by LIM kinase (LIMK) (Song et al. 2006; van Rheenen et al. 2007). LIMK is activated nearly simultaneously with PLC (Song et al. 2006) through Rho/Rho associated kinase (ROCK) and Rac/p21-activated kinase (PAK)-dependent pathway (Dan et al. 2001). Thus the translocation of cofilin from the plasma membrane to the F-actin compartment is induced by PLC-mediated decrease in $PIP_2$ levels, whereas the translocation from the cytosol to the plasma membrane compartment is induced by dephosphorylation of cofilin (van Rheenen et al. 2009). In mammary-tumor cells the reduction in $PIP_2$ levels by PLC, with the subsequent release of cofilin, is the key to regulating cofilin activity after receptor stimulation (Mouneimne et al. 2004). By contrast, in leukocytes, the primary activation of cofilin is through dephosphorylation (Boldt et al. 2006; Sun et al. 2007) although the binding of $PIP_2$ might be the key to regulating the activity locally at the cell periphery.

$PIP_2$ plays also an important role in focal adhesion formation. It binds and regulates the function of proteins that link the extracellular matrix to actin filaments via integrins. $PIP_2$ binds to vinculin and promotes its binding to actin filaments and to talin (Gilmore and Burridge 1996; Huttelmaier et al. 1998). The interaction between actin cytoskeleton and plasma membrane not only modulates local adhesion energy but also influences cell shape and migration. The ERM family members link actin filaments and surface glycoproteins, and this association is enhanced by $PIP_2$. ERM proteins are regulated by $PIP_2$ in synergy with Rac since they are effectors of this small GTP-binding protein (Hirao et al. 1996; Mackay et al. 1997). Similarly, an effector of Cdc42, WASP, binds $PIP_2$ (Miki et al. 1996). $PIP_2$ also interacts with the PKC substrates, plekstrin and MARCKS, and promotes their association with actin cytoskeleton (Glaser et al. 1996; Harlan et al. 1995).

### *6.2.2 Regulation by the Small GTP-binding Proteins: Rho, Rac and Cdc42*

Small GTP-binding proteins of the Rho family belong to the Ras superfamily and are intracellular signaling proteins known to act as molecular switches to control actin cytoskeleton dynamics. The best characterized members: RhoA, Rac1, and Cdc42 were first identified in the early 1990s (Ridley and Hall 1992). They play an essential role in the regulation of various cellular processes, including cell migration, smooth muscle contraction, endocytosis, cytokinesis, neurite outgrowth and retraction. They are conformationally regulated by the binding of GTP and GDP. The active form of Rho is GTP-bound (Hall 1998; Van Aelst and D'Souza-Schorey 1997). The exchange of GDP by GTP, catalyzed

by guanine nucleotide exchange factors (GEFs), allows Rho proteins to interact with and activate their downstream effectors and thereby transmit signals (Schmitz et al. 2000) (see Chap. 7). It is established that activation of Rho GTPases is controlled by several mechanisms including the stimulation of receptor tyrosine kinases and that many among the G protein-coupled receptors (GPCRs), particularly those coupled to the $G_{12}$, $G_o$ and $G_q$ type of heterotrimeric G proteins, are upstream regulators of the Rho proteins. Rac activation by both tyrosine kinases and G-coupled receptors is dependent on phosphatidylinositol 3-kinase (PI3K) activity (Royal et al. 2000). During lamellipodia extension phosphoinositol phosphates also bind and activate GEFs that regulate the activity of Rac (Kaibuchi et al. 1999). Rho GTPase downstream target proteins include protein kinases, lipid-modifying enzymes and activators of the Arp2/3 complex (Etienne-Manneville and Hall 2002).

The ability of a cell to move requires an asymmetrical localization of cellular activities. The front of the migrating cell generates a protrusive force, generally associated with the extension of a lamellipodium and development of new cell adhesions to the extracellular substrate. Cell contractility is required to allow cell body to follow the extending front (Ridley 2001; Worthylake et al. 2001). Through its ability to promote both protrusion and cell contraction, the actin cytoskeleton provides the driving force for cell migration. RhoA, Rac1 and Cdc42 proteins are the key regulators of actin cytoskeleton dynamics involved in cell shape changes, protrusion, adhesion and contractility (Clark et al. 1998; Nobes and Hall 1995, 1999). Distinctive effects of Rho, Rac, and Cdc42 activation on the organization of the actin cytoskeleton have been observed in many cell types, including fibroblasts, macrophages, epithelial cells, endothelial cells, astrocytes, neurons and in cancer cells as well as in circulating cells such as lymphocytes, mast cells and platelets (Allen et al. 1997; Hartwig et al. 1995; Ridley et al. 1995).

Cdc42 is the principal determinant in establishing cell polarity and stabilization of the directional movement (chemotaxis) (Itoh et al. 2002). Rac and Cdc42 regulate also small integrin clusters at the leading edge, known as focal complexes, that stabilize the lamellipodium (Ridley et al. 2003). Rac and Cdc42 induce Arp2/3 complex- mediated actin polymerization in an area limited to the cell protrusion and thus enable directional migration. When the Arp2/3 complex is bound with proteins of the WASP or WAVE family, it is activated and catalyzes nucleation of actin polymerization and the formation of new filament branches (Condeelis 2001; Pollard et al. 2000). The product of the human Wiskott-Aldrich syndrome gene, WASP, has been identified as a Cdc42-specific target (Aspenstrom 1997; Welch and Mullins 2000). WAVE is itself regulated by Rac (Cory and Ridley 2002; Ridley 2001). Rac interacts with the insulin receptor tyrosine kinase substrate p53 (IRSp53) (Miki et al. 2000), which in turn interacts through an Src-homologous domain 3 (SH3) with a number of the WAVE family proteins, which then bind to and activate the Arp2/3 complex. An active Arp2/3 complex binds to proteins called nucleation promoting factors (Campellone and Welch 2010; Chesarone and Goode 2009). Another target of Rac that may be involved in actin polymerization is PIP 5-kinase, the enzyme that converts phosphoinositol phosphate (PIP) to $PIP_2$ (Hartwig et al. 1995). Rac can also control the activity of a crucial regulator of actin assembly, cofilin. Through its effector, PAK, which phosphorylates and activates LIM kinase, Rac regulates LIMK activity (Dan et al. 2001; Edwards et al. 1999; Maekawa et al. 1999; Yang et al. 1998). RhoA-mediated reorganization of the filamentous actin network involves RhoA downstream effectors, among them, the Rho family of serine-threonine protein kinases (ROCKs) that, in turn, affect their specific substrate proteins (Schmitz et al. 2000; Tsuji et al. 2002). ROCK acts upon LIM kinase and testis-specific kinase (TESK), which phosphorylate cofilin at Ser 3 (Arber et al. 1998; Bernard 2007; Maekawa et al. 1999; Pak et al. 2008). LIMK phosphorylates and inactivates cofilin in the cytosol (Delorme et al. 2007). Cofilin ability to bind actin, and therefore its actin severing and depolymerizing activity is inhibited upon phosphorylation (Bamburg et al. 1999; DesMarais et al. 2005). Dephosphorylation of cofilin by the phosphatase Slingshot homolog (SSH) and chronophin (CIN) (Huang et al. 2006; Niwa et al. 2002; Ohta et al. 2003) circumvents this inhibition. Cofilin can increase the number of barbed ends by severing the existing microfilaments (Ichetovkin et al. 2002) and by its actin-nucleation activity (Andrianantoandro and

Pollard 2006). The actin-depolymerization activity of cofilin increases the pool of actin monomers available for polymerization (van Rheenen et al. 2009).

A number of myosins have been implicated in cell migration (Mermall et al. 1998). Myosin light chain phosphorylation is enhanced in the lamellipodial region of cells (Matsumura et al. 1998), which suggests a role for myosins in lamellipodium extension. Rac can affect the phosphorylation of both myosin heavy chains (MHC) (van Leeuwen et al. 1999) and myosin light chains via activation of its downstream kinase, PAK (Brzeska et al. 2004; Klosses et al. 2001). In polarized cells Rac activity is constrained to the cell front. FRET (fluorescence resonance energy transfer) microscopy has revealed that Rac GTP levels are highest at the leading edge of a migrating cell (Itoh et al. 2002). Rho GTP is restricted to the cell body and excluded from the leading edge (Worthylake et al. 2001). Coordination of localized actin polymerization at the front of the cell with contraction at the rear can propel cells forward.

Cell body contraction is dependent on acto-myosin contractility. RhoA plays an important role in contractile force generation needed for the assembly of stress fibers (Fukata et al. 2001), and large integrin clusters – focal adhesions (Ridley et al. 2003) as well as tail retraction, which is a myosin dependent process (Worthylake et al. 2001). ROCK affects the acto-myosin II contractility by phosphorylation and inhibition of the myosin-binding subunit of myosin light chain phosphatase (MLCP) at Thr853 and Thr696 (Hartshorne et al. 1998; Kawano et al. 1999; Kimura et al. 1996; Ramachandran et al. 2011). The actin-activated ATPase activity of myosin II is also increased by direct phosphorylation of MLC at Ser19 (Amano et al. 1996; Matsumura 2005; Moussavi et al. 1993; Riento and Ridley 2003). The ROCK-mediated activation of myosin II is suggested to cause assembly of stress fibers and focal adhesions in nonmuscle cells (Chrzanowska-Wodnicka and Burridge 1996; Kawano et al. 1999; Kimura et al. 1996). The RhoA/ROCK-mediated increase in the level of phosphorylated MLC and contraction occurs in the absence of $Ca^{2+}$ (Kimura et al. 1996) while myosin light chain kinase phosphorylates MLC at Ser19 and/or Thr18 residues in a $Ca^{2+}$-dependent way (Katoh et al. 2001; Somlyo and Somlyo 2003). Accordingly, G protein-coupled receptors (GPCRs) that mobilize intracellular-free $Ca^{2+}$ activate MLCK and induce MLC phosphorylation.

These two kinases, ROCK and MLCK, may play distinct roles in the spatial regulation of myosin II activity. The RhoA/ROCK signaling pathway plays an important role in generating myosin II-based contractility and stress fiber assembly in the center of cells (Chrzanowska-Wodnicka and Burridge 1996; Kawano et al. 1999; Kimura et al. 1996; Totsukawa et al. 2000). $Ca^{2+}$/calmodulin MLCK regulation is primarily responsible for phosphorylating MLC at the cell periphery (Totsukawa et al. 2000). A reduction in Rho or Rho-kinase activity affects morphology, motility and invasion of all cell types examined until now.

Another RhoA effector, mDia1, induces actin polymerization and cooperates with ROCK in stress fiber formation (Watanabe et al. 1999). Although not direct targets of Rho, the ERM proteins are essential for Rho- and Rac-induced cytoskeletal effects. Their interaction with a transmembrane protein, CD44, and filamentous actin can be regulated by Rho (Fukata et al. 1998; Hirao et al. 1996; Mackay et al. 1997; Matsui et al. 1998).

Axon guidance can be thought of as a specialized form of directed migration: the cell body does not move, but the growth cone at the axon tip responds to attractive and repulsive extracellular cues (Pak et al. 2008). Cdc42 and Rac are positive regulators of neurite outgrowth, whereas RhoA inhibits neurite extension (Luo 2000). Rac activation induces growth cone advance and increases the number of dendritic spines, RhoA has the opposite effect (Wong et al. 2000). Axonal extension is driven by actin polymerization within the growth cone consisting of filopodial and lamellipodial protrusions. Activation of Rac and Cdc42 in neuroblastoma cells has been shown to promote the formation of lamellipodia and filopodia, respectively, along the neurite extensions (Luo 2000).

The assembly and disassembly of peripheral actin filaments can be utilized to promote localized changes in the structure of the plasma membrane which subsequently affect membrane-controlled processes such as phagocytosis and pinocytosis. Two distinct signaling pathways regulate phagocytosis.

When induced by immunoglobulin receptor stimulation, phagocytosis requires Rac and Cdc42, and when induced by the complement receptor it requires Rho (Caron and Hall 1998).

In addition to their effect on migration and endocytosis, Rho GTPases contribute to the regulation of the mitotic phase of the cell cycle. Mitotic cell rounding is the process of cell shape change. In many types of cells the rearrangement of the actin cytoskeleton and an increase in cortical rigidity that accompany mitotic cell rounding require RhoA and Rac1 (Glotzer 2001; Maddox and Burridge 2003). The cell cycle is completed with cytokinesis, and in animal cells this is driven by an actin and myosin contractile ring, which constricts to form the two daughter cells. Inhibition of Rho as well as expression of constitutively activated Rho prevents the assembly of the contractile ring in a variety of mammalian cells (Kosako et al. 2000). Activated Rho recruits ROCK and citron kinase and probably also the Diaphanous-related proteins, to assemble the contractile ring, mediate cleavage furrow ingression and promote abscission (Eda et al. 2001; Gruneberg et al. 2006; Kosako et al. 2000; Madaule et al. 1998; Piekny et al. 2005). ROCK is responsible for phosphorylation of MLC in the cleavage furrow. Citron kinase phosphorylates MLC at both Ser19 and Thr18 and increases diphosphorylation of MLC during cytokinesis. Both kinases accumulate at the cleavage furrow in several types of cultured cells (Eda et al. 2001; Yamashiro et al. 2003).

## 6.3 Compensation of ROCK Inhibition by P2Y$_2$R Activated Signaling Pathways

Blocking of the RhoA/ROCK signaling pathway in glioma C6 cells causes serious changes in the organization of actin cytoskeleton, cell shape and migration. These changes can be compensated by UTP stimulation of the P2Y$_2$R. Recovery of the cells from ROCK inhibition requires P2Y$_2$R coupling with both G$_q$ and G$_o$ proteins. Induction of G$_o$-mediated signal transduction depends on $\alpha_v\beta_5$ integrin activation.

### *6.3.1 ROCK Inhibition in Glioma C6 Cells*

Addition of a specific inhibitor of ROCK – Y-27632 inhibits stress fibers and focal adhesions in several cell lines. In glioma C6 cells ROCK inhibition induces changes in F-actin organization and cell shape similar to those observed in other types of cells with blocked RhoA/ROCK signaling pathway.

Our results have shown that actin reorganization and subsequent alterations in glioma C6 cell morphology and migration correlate with the level of phosphorylated MLC (P-MLC) and phosphorylated cofilin (P-cofilin) – two actin binding proteins downstream of RhoA/ROCK. ROCK inhibition by Y-27632 reduces the level of P-MLC (Fig. 6.1a) (Korczyński et al. 2011), the level of P-cofilin and simultaneously increases the level of Rac1 GTP (Fig. 6.1b, unpublished results) (Korczyński et al. in preparation). The observed activation of Rac1 following ROCK inhibition may result from the influence of Rho on Rac, as it has been observed in neurons (Kozma et al. 1997; van Leeuwen et al. 1997), Swiss 3T3 cells (Tsuji et al. 2002) and in malignant astrocytoma (Salhia et al. 2005). Tsuji and coworkers have revealed that the Rho-dependent Rac activation is mediated by mDia1 through Cas phosphorylation (Tsuji et al. 2002).

Glioma C6 cells normally exhibit a polygonal shape and are well spread on the substrate. They are usually characterized by a fibroblast-like morphology (Fig. 6.2a) (see Chap. 3). Actin filaments form a cortical network just beneath the plasma membrane and stress fibers in the cytoplasm. Phosphomyosin II is distributed along the stress fibers and under the cell membrane (Fig. 6.2b). Cells are polarized with clearly defined lamellipodium. Decreasing the level of phosphorylated MLC and

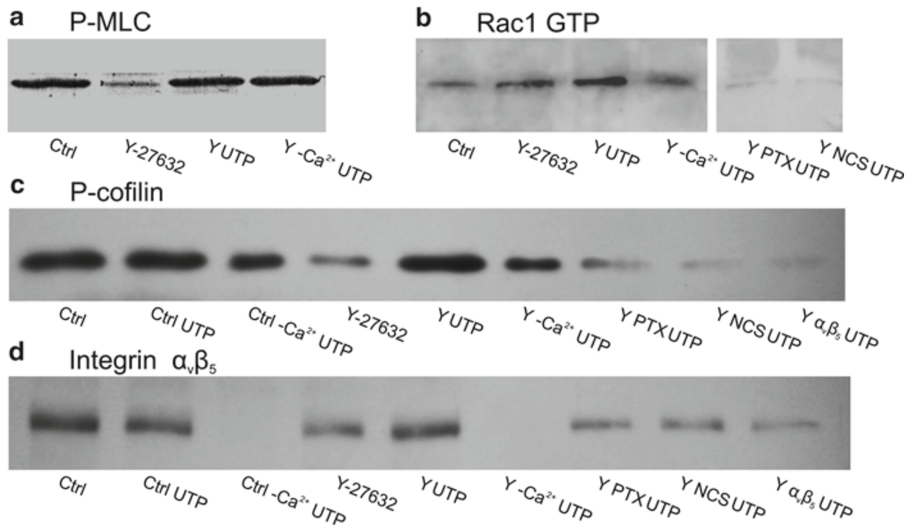


**Fig. 6.1** Changes in protein phosphorylation level in glioma C6 cells after ROCK inhibition with Y-27632. Western blot analysis of (**a**) P-MLC, (**b**) Rac1 GTP, (**c**) P-cofilin and (**d**) Integrin $\alpha_v\beta_5$ level in cells treated with different reagents before material collection. Abbreviations for experimental models: **Ctrl –** control cells, **Ctrl UTP –** cells stimulated with UTP, **Ctrl -Ca²⁺ UTP –** control cells stimulated with UTP in calcium-free medium, **Y-27632** – cells pretreated with Y-27632 inhibitor, **Y UTP** – UTP stimulated Y-27632-pretreated cells, **Y -Ca²⁺ UTP** – Y-27632 pretreated cells stimulated with UTP in calcium-free medium, **Y PTX UTP** – cells pretreated with Y-27632 and pertussis toxin (PTX) stimulated with UTP, **Y ML-7 UTP** – Y-27632 and ML-7-pretreated cells followed by UTP stimulation, **Y NCS UTP** – Y-27632 and NCS 23766-pretreated cells stimulated with UTP, **Y $\alpha_v\beta_5$ UTP** – cells pretreated with Y-27632 and $\alpha_v\beta_5$ integrin antibody followed by UTP stimulation

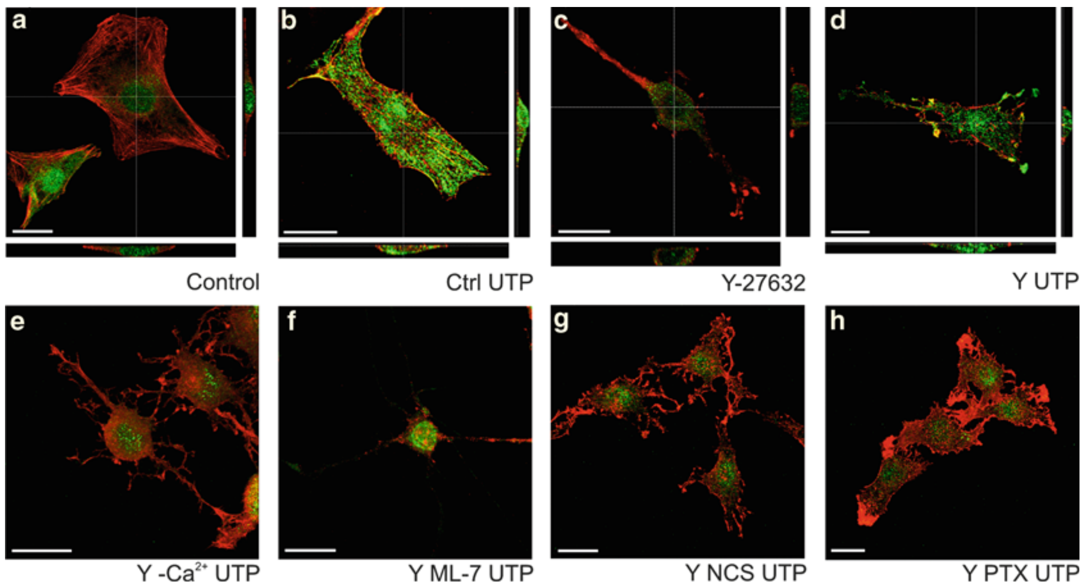


**Fig. 6.2** Changes in morphology of glioma C6 cells in different experimental models (for abbreviations see Fig. 6.1). 3D orthogonal sections (**a–d**) and single images (**e–h**) from confocal microscopy. Immunofluorescence staining for F-actin (*red*) and P-cofilin (images **a**, **c**, **e**, **g**, **h**; *green*) or P-MLC (images **b**, **d**, **f**; *green*). Scale bars 20 µm

cofilin results in the loss of cell polarization, in stress fibers decomposition and promotes membrane ruffling and multiple lamellipodia formation. The cells round up and develop long outgrowths. Rounded cells exhibit a high volume to surface ratio as the 3D images show (Fig. 6.2c). F-actin is concentrated mainly under the cell surface and in the elongated processes.

Myosin II inactivation in the presence of Y-27632 contributes to destabilization of the actin cytoskeleton. Since the F- to G-actin ratio is not affected under such conditions (Targos et al. 2006) changes observed in actin cytoskeleton organization (stress fiber disappearance, relocation of F-actin) and in cell morphology seem to be caused by disorganization of the functional state of acto-myosin II system by inhibition of Rho-kinase. Dephosphorylation of MLC would favor myosin remaining in the low-affinity state for actin and subsequently dissociating from actin (Sutton et al. 2001). Phosphorylated myosin II diffuses all over the rounded cell body and does not co-localize with actin filaments. Temporal separation between myosin II inactivation and a complete disruption of stress fibers (Sutton et al. 2001) suggests that other processes, such as actin severing, may be involved in stress fiber disassembly. In glioma C6 cells suppression of LIMK-mediated phosphorylation of cofilin might be responsible for microfilament destabilization in the cell center. However, the ability of cofilin to affect actin dynamics is restricted at the leading edge because in this region microfilaments are not saturated with tropomyosin and not resistant to cofilin severing (DesMarais et al. 2002; Gupton et al. 2005). Changes in the actin cytoskeleton organization described above, together with the dysfunction of cell adhesion regulation, impair directional cell migration. Human TM cells treated with ROCK inhibitors showed a decrease in the electrical cell-substrate resistance and a decrease in tyrosine phosphorylation of paxilin and focal adhesion kinase (FAK) (Ramachandran et al. 2011).

### *6.3.2 Effect of P2Y$_2$ Receptor Stimulation on MLC Phosphorylation – The Role of MLCK*

In glioma C6 cells UTP stimulation induces an about 15 % increase in the pool of P-MLC. However, it does not cause visible changes in F-actin distribution and cell morphology even though P2Y$_2$R promotes the formation of stress fibers (Fig. 6.2b) and cell migration (Korczyński et al. 2011) similarly as in astrocytoma cells (Peterson et al. 2010). Moreover stimulated glioma C6 cells are more spread than the control ones. In cells pretreated with ROCK inhibitor, stimulation of P2Y$_2$R induces almost a 65 % increase in P-MLC level (Fig. 6.1a) (Korczyński et al. 2011). The relatively lower level of P-MLC after UTP stimulation in cells with inhibited ROCK, as compared to control cells, seems to be the result of constitutively active myosin II phosphatase which is inhibited by ROCK under normal conditions.

UTP stimulation of Y-27632 pretreated glioma C6 cells correlates with a dynamic actin cytoskeleton reorganization and subsequent recovery of control cell-like morphology (Fig. 6.2d). Myosin II phosphorylation occurs rapidly, and temporally correlates with the assembly of short, weak stress fibers at the cell periphery (Fig. 6.2d). Stress fiber localization and the fact that myosin II phosphorylation follows G$_q$-mediated calcium mobilization indicates that Ca$^{2+}$/calmodulin dependent myosin light chain kinase may be responsible for the phosphorylation of MLC under such experimental conditions. It was shown that in cells with blocked ROCK and activated MLCK stress fibers are not assembled in the cell center (Katoh et al. 2001; Totsukawa et al. 2000). MLCK inhibition by a specific inhibitor, ML-7, almost completely prevented MLC phosphorylation in Y-27632 pretreated glioma C6 cells and in consequence made actin cytoskeleton reorganization and cell recovery impossible (Fig. 6.2f) (Korczyński et al. 2011). Similar results concerning MLCK inhibition were obtained also for non-transformed astrocytes (Baorto et al. 1992).

Apart from calcium, other upstream messengers may participate in the regulation of MLCK after $P2Y_2$ stimulation. Active Rac, for example, may inhibit MLCK at the leading edge (Brzeska et al. 2004; Sanders et al. 1999). The regulatory mechanism could be complex and the activity of MLCK probably vary depending on the cell type. The Rac/PAK signaling pathway is also involved in direct phosphorylation of myosin light chains (Brzeska et al. 2004).

### *6.3.3 Effect of $P2Y_2$ Receptor Stimulation on Cofilin Phosphorylation – The Role of Rac1 Protein*

Our results have also shown the effect of UTP on cofilin phosphorylation in glioma C6 cells. The $P2Y_2$ receptor activation significantly increases the level of phosphorylated cofilin as well as that of Rac1 GTP in cells with blocked ROCK (Fig. 6.1b). It has been established that activated Rac increases association of PAK with LIMK (Edwards et al. 1999) and in consequence enhances the activity of LIMK (Yang et al. 1998). Signaling pathways which negatively regulate cofilin in glioma C6 cells stimulated with UTP are unknown. We suggest that activation of LIMK by Rac1/PAK signaling pathway may be responsible for cofilin phosphorylation in glioma C6 cells with blocked ROCK, and that activation of Rac1 protein is mediated by $G_o$ protein. The level of phosphorylated cofilin is not increased in cells pretreated with NCS 23766 (Rac1 inhibitor) or with pertussis toxin (PTX) – $G_{i/o}$ inhibitor (Fig. 6.1c). Under such experimental conditions the recovery from ROCK inhibition is not completed (Fig. 6.2g, h) although $G_q$-mediated intracellular calcium mobilization and myosin II activation is not affected. The complete reversal of the effects of ROCK inhibition in glioma C6 cells requires induction of $G_q$ and $G_o$ mediated signaling pathways and subsequent increasing the level of P-MLC as well as phosphorylated cofilin (Korczyński et al. in preparation). However, in mammary-tumor cells the phosphorylation status of cofilin is not directly related to cofilin activity, and actin depolymerization depends on PLC activity but not on cofilin dephosphorylation. (Mouneimne et al. 2004; van Rheenen et al. 2009; Song et al. 2006). In carcinoma cells P-cofilin level rises upon EGF stimulation while the actin-severing activity of cofilin is at its highest level (Song et al. 2006). If so, cofilin dephosphorylation in glioma C6 cells with blocked ROCK should not be identify with its ability to actin binding. Dephosphorylated cofilin is immediately bound by $PIP_2$ and inactivated (Rheenen et al. 2009). The morphological effect of decreasing the level of phosphorylated cofilin in glioma C6 cells with blocked ROCK may be compared to suppression of cofilin in MTC cells, a non-metastatic mesenchymal-type tumor cells with high polarity and movement (Shestakova et al. 1999), that caused changes in the cell shape, the formation of numerous lamellipodia and subsequent decrease in the directionality of migration (Sidani et al. 2007). This may suggests that the activity status of cofilin in glioma C6 cells does not only depend on phosphorylation-dephosphorylation processes, and that in cells with blocked ROCK the level of cofilin activity is suppressed.

Stimulation of $P2Y_2$ receptor, *via* $G_q$ protein, activates PLC and a subsequent decrease in the level of $PIP_2$. Cofilin molecules are released and diffuse to the cortical layer, where cofilin binds and severs actin filaments to force actin polymerization (van Rheenen et al. 2007). However, cofilin activation by PLC is not sufficient for glioma C6 cells recovery from ROCK inhibition since blocking the Rac1 activity prevents from this process. Simultaneously with PLC activation of LIMK through Rac1/PAK-dependent pathway phosphorylates and inactivates cofilin molecules that diffuse away from the plasma membrane (Delorme et al. 2007). Cofilin inactivation together with myosin II activation through calcium dependent MLCK and Rac/PAK signaling pathways (Brzeska et al. 2004) cause actin cytoskeleton reorganization leading to stress fiber assembly in the cell periphery, lamellipodium formation, alterations in cell morphology and migration. It is also possible that UTP stimulation of glioma C6 cells induces $G_{12}$-mediated RhoA activation similar to that observed in human astrocytoma cells (Liao et al. 2007). This in turn activates a downstream effector

mDia1 by disrupting its intermolecular interactions. Active mDia1 induces actin polymerization and the formation of thin actin stress fibers, which however are disorganized when ROCK is inhibited (Watanabe et al. 1999).

### 6.3.4 *Effect of Integrin on Glioma C6 Cell Recovery from ROCK – Essential Role of Rac1 Protein*

The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins play essential roles in migration of numerous cell types (see Chap. 7). In astrocytes $P2Y_2R$ interaction with $\alpha_v\beta_5$ integrin increases chemotactic and chemokinetic cell response. UTP stimulation also increases the expression of $\alpha_v\beta_{3/5}$ integrins in astrocytes (Wang et al. 2005). Activation of $\alpha_v\beta_5$ integrin in UTP stimulated glioma C6 cells (Fig. 6.1d, unpublished results) is important for cell spreading and for coupling $P2Y_2R$ to $G_o$. $G_o$-mediated Rac1 activation thus requires interaction of $P2Y_2R$ with $\alpha_v\beta_5$ integrin and is crucial for morphology remodeling of cells with blocked ROCK (Korczyński et al. in preparation). In calcium-free medium, in detached cells and in cells pretreated with anti-$\alpha_v\beta_5$ antibody integrins are not activated (Fig. 6.1d), and their interaction with $P2Y_2Rs$ and in consequence coupling the $P2Y_2R$ to $G_o$ protein is blocked. Under such experimental conditions Rac1 activation and subsequent Rac1-mediated cofilin phosphorylation is inhibited. The morphological effect of blocking $\alpha_v\beta_5$ integrin is similar to that of ROCK inhibition through Y-27632 (Korczyński et al. in preparation).

Under UTP stimulation of glioma C6 cells, the $P2Y_2$ receptor initiates a typical store operated calcium signal (Suplat-Wypych et al. 2010). This signal pathway enhances MLC phosphorylation and, in cells with blocked ROCK, temporally correlates with renormalization (Fig. 6.2d). However, if UTP stimulation is performed in calcium-free medium, and the calcium response is limited to $Ca^{2+}$ release from intracellular stores, actin cytoskeleton reorganization and the morphology recovery do not take place even though MLC phosphorylation facilitates cell contractility as manifested by cell spreading measured by confocal imaging (Fig. 6.2e). MLC phosphorylation is not sufficient for the reversal of cells with blocked ROCK to their normal phenotype. For cell recovery from ROCK inhibition two signaling pathways must be triggered: $G_q$-mediated PLC activation for mobilization of intracellular calcium and cofilin release from $PIP_2$ and $G_o$-mediated Rac1 activation necessary for cofilin phosphorylation as well as integrin activation (Fig. 6.3). Rac and Cdc42 regulate small integrin clusters at the leading edge known as focal complexes that stabilize the lamellipodium (Ridley et al. 2003). In UTP stimulated glioma C6 cells with blocked ROCK, $\alpha_v\beta_5$ integrins are activated by Rac1. Inhibition of positive Rac1 regulation by NCS or PTX block integrins activation (Fig. 6.1d) and cell adhesion as well as cofilin phosphorylation and cell polarization. Together with suppression of cofilin phosphorylation prevents glioma C6 cells from recovery of control cell like morphology.

## 6.4 Summary

Central nervous system glial cells release and respond to nucleotides under both physiological and pathological conditions, suggesting that these molecules play key roles in both normal brain function and in repair after damage. Signaling cascades evoked by $P2Y_2$ receptor plays an important role in the phenomena dependent on the actin cytoskeleton dynamics endocytosis, cell division, adhesion, intracellular transport and migration. In astrocytes and microglial cells the agonists activate $P2Y_2Rs$ to induce integrin-dependent activation of Rho and Rac, promoting glial cells migration. Thus, signaling pathways related to $P2Y_2Rs$ are of special interest in glioma tumor cells, where cytoskeleton driven cell motility is the main way of cancer invasion in brain.

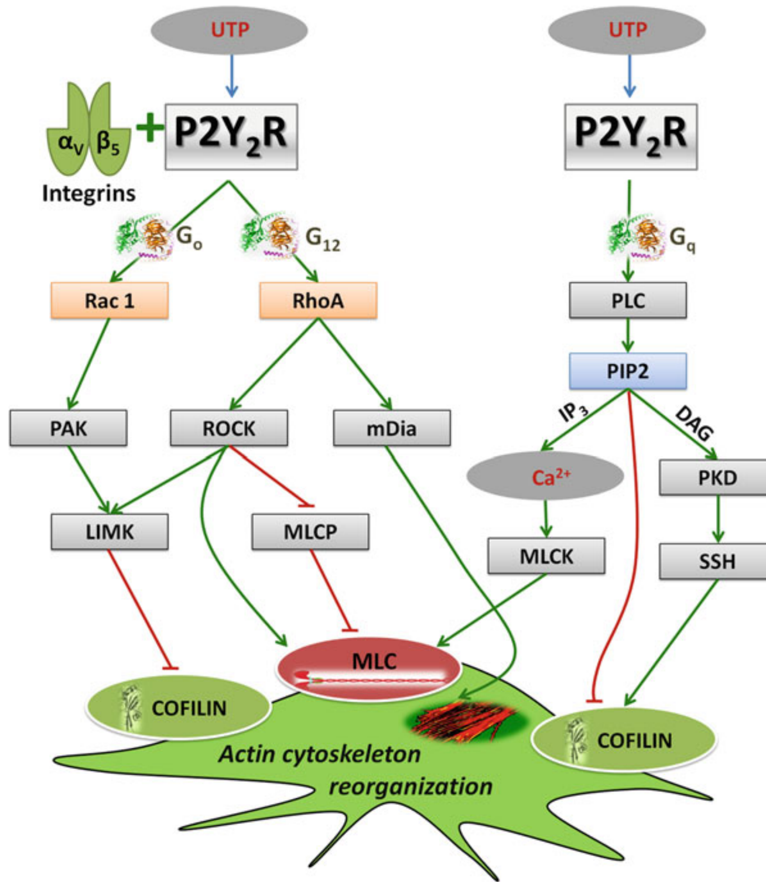


**Fig. 6.3** Schematic diagram of signaling pathways activated by external UTP stimulation of $P2Y_2$ receptor in glioma C6 cells. *Green arrows* on the scheme indicate activation of downstream substrates, *red arrows* show inactivation of cofilin or MLC. For details, see text

UTP stimulation of $P2Y_2R$ in glioma C6 cells compensates changes in the organization of actin cytoskeleton, cell shape and migration caused by blocking of the RhoA/ROCK signaling pathway. The complete renormalization of glioma C6 cells with blocked ROCK requires: (1) induction of $G_q$-mediated PLC activation and mobilization of intracellular calcium for increasing the level of P-MLC, (2) cofilin release from $PIP_2$, (3) $G_o$-mediated Rac1 activation necessary for both cofilin phosphorylation and integrin activation. Induction of $G_o$-mediated signal transduction depends on $\alpha_v\beta_5$ integrins activation (Korczyński et al. in preparation). In calcium-free medium, as well as in detached cells, UTP stimulation of glioma C6 cells with blocked ROCK does not cause $\alpha_v\beta_5$ integrins activation, blocking the interaction between $P2Y_2Rs$ and $\alpha_v\beta_5$ integrins and in consequence coupling the $P2Y_2R$ to $G_o$ protein. For cell recovery from ROCK inhibition $\alpha_v\beta_5$ integrins activation is necessary for signal transduction and focal complexes formation that stabilize new formed lamellipodium, fix cell polarization and directional migration. In UTP stimulated cells with blocked ROCK, $\alpha_v\beta_5$ integrins are activated by Rac1. Inhibition of positive Rac1 regulation by NCS or PTX decreases integrins activation and cell adhesion and together with suppression of cofilin phosphorylation prevents glioma C6 cells to complete recovery of control cell like morphology. Inhibition of this signaling pathway can also affect directional migration of the cell as cofilin activity is necessary to induce protrusions and to define the direction of cell motility (Ghosh et al. 2004).

Since the RhoA/ROCK signaling pathway is crucial for migration, infiltrative growth and expansion of brain tumors, the attempts are made to inhibit the development of this type of cancer by inhibition of RhoA or ROCK. However numerous publications mentioned in this chapter indicate a high possibility of compensation of this inhibition by alternative signaling pathways especially related to cell stimulation by nucleotides. This fact can significantly impede the use of currently known inhibitors in medical therapies of brain tumors.

## References


Allen WE, Jones GE, Pollard JW, Ridley AJ (1997) Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. J Cell Sci 110:707–720

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K (1996) Pohsphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271:20246–20249

Andrianantoandro E, Pollard TD (2006) Mechanism of actin filament turnover by severing and nucleation at different concentrations of ADF/cofilin. Mol Cell 24:13–23

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393:805–809

Aspenstrom P (1997) A Cdc42 target protein with homology to the non-kinase domain of FER has a potential role in regulating the actin cytoskeleton. Curr Biol 7:479–487

Bagchi S, Liao Z, Gonzales FA, Chorna NE, Seye CI, Weisman GA, Erb L (2005) The P2Y2 nucleotide receptor interacts with alpha integrins to activate Go and induce cell migration. J Biol Chem 280:39050–39057

Bamburg JR, McGough A, Ono S (1999) Putting a new twist on actin: ADF/cofilin modulate actin dynamics. Trends Cell Biol 9:364–370

Baorto DM, Mellado W, Shelanski ML (1992) Astrocyte process growth induction by actin breakdown. J Cell Biol 117:357–367

Bernard O (2007) Lim kinases, regulators of actin dynamics. Int J Biochem Cell Biol 39:1071–1076

Bishop AL, Hall A (2000) Rho GTPases and their effector proteins. Biochem J 348:241–255

Boarder MR, Hourani SM (1998) The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol Sci 19:99–107

Boldt K, Rist W, Weiss SM, Weith A, Lenter MC (2006) FPRL-1 induces modifications of migration-associated proteins in human neutrophils. Proteomics 6:4790–4799

Brzeska H, Szczepanowska J, Matsumura F, Korn ED (2004) Rac-induced increase of phosphorylation of myosin regulatory light chain in HeLa cells. Cell Motil Cytoskel 58:186–199

Campellone KG, Welch MD (2010) A nucleator arms race: cellular control of actin assembly. Nat Rev Mol Cel Biol 11:237–251

Caron E, Hall A (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science 282:1717–1721

Chesarone MA, Goode BL (2009) Acti nucleation and elongation factors: mechanisms and interplay. Curr Opin Cell Biol 21:28–37

Chrzanowska-Wodnicka M, Burridge K (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 133:1403–1415

Chung CY, Funamoto S, Firtel RA (2001) Signaling pathways controlling cell polarity and chemotaxis. Trends Biochem Sci 26:557–566

Clark EA, King WG, Brugge JS, Symons M, Hynes RO (1998) Integrin-mediated signals regulated by members of the Rho family of GTPases. J Cell Biol 142:573–586

Condeelis J (2001) How is actin polymerization nucleated in vivo? Trends Cell Biol 11:288–293

Cooper JA, Schafer DA (2000) Control of actin assembly and disassembly at filament ends. Curr Opin Cell Biol 12:97–103

Cory GO, Ridley AJ (2002) Cell motility: braking WAVEs. Nature 418:732–733

Dan C, Kelly A, Bernard O, Minden A (2001) Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. J Biol Chem 276:32115–32121

Delorme V, Machacek M, DerMardirossian C, Anderson KL, Wittmann T, Hanein D, Waterman-Storer C, Danuser G, Bokoch GM (2007) Cofilin activity downstream of Pak1 regulates cell protrusion efficiency by organizing lamellipodium and lamella actin network. Dev Cell 13:646–662

DesMarais V, Ichetovkin I, Condeelis J, Hitchcock-DeGregori SE (2002) Spatial regulation of actin dynamics: a tropomyosin-free, actin-rich compartment at the leading edge. J Cell Sci 115:4649–4660

DesMarais V, Ghosh M, Eddy R, Condeelis J (2005) Cofilin takes the lead. J Cell Sci 118:19–26
Eda M, Yonemura S, Kato T, Watanabe N, Ishizaki T, Madaule P, Narumiya S (2001) Rho-dependent transfer of Citron-kinase to the cleavage furrow of dividing cells. J Cell Sci 114:3273–3284
Edwards DC, Sanders LC, Bokoch GM, Gill GN (1999) Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signaling to actin cytoskeleton dynamics. Nat Cell Biol 1:253–259
Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Pérez LI, González FA, Gresham HD, Turner JT, Weisman GA (2001) An RGD sequence in the $P2Y_2$ receptor interacts with $\alpha_v\beta_3$ ntegrins and is required for $G_o$-mediated signal transduction. J Cell Biol 153:491–501
Erb L, Liao Z, Seye CI, Weismann GA (2006) P2 receptors: intracellular signaling. Eur J Physiol 452:552–562
Etienne-Manneville S, Hall A (2002) RhoGTPases in cell biology. Nature 420:629–635
Fukami K, Furuhashi M, Inagaki M, Endo T, Hatano S, Tekanawa T (1992) Requirement of phosphatidylinositol 4,5-biphosphate for alpha-actinin function. Nature 359:150–152
Fukata Y, Kimura K, Oshiro N, Saya H, Matsumura Y, Kaibuchi K (1998) Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. J Cell Biol 141:409–418
Fukata Y, Amano M, Kaibuchi K (2001) Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol 22:32–39
Ghosh M, Song X, Mouneimne G, Sidani M, Lawrence DS, Condeelis JS (2004) Cofilin promotes actin polymerization and defines the direction of cell motility. Science 304:743–746
Glaser M, Wanaski S, Buser CA, Boguslavsky V, Rashidzada W, Morris A, Rebecchi M, Scarlata SF, Runnels LW, Prestwich GD, Chen J, Aderem A, Ahn J, McLaughlin S (1996) Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. J Biol Chem 271:26187–26193
Gilmore AP, Burridge K (1996) Regulation of vinculin binding to talin and actin by phosphatidylinositol 4,5-biphosphate. Nature 381:531–535
Glotzer M (2001) Animal cell cytokinesis. Annu Rev Cell Dev Biol 17:351–386
Greig AV, James SE, McGrouther DA, Terenghi G, Burnstock G (2003) Purinergic receptor expression in the regeneration epidermis in a rat model of normal and delayed wound healing. Exp Dermatol 12:860–871
Gruneberg U, Neef R, Li X, Chan EH, Chalamalasetty RB, Nigg EA, Barr FA (2006) KIF14 and citron kinase act together to promote efficient cytokinesis. J Cell Biol 172:363–372
Gupton SL, Anderson KL, Kole TP, Fischer RS, Ponti A, Hitchcock-DeGregori SE, Danuser G, Fowler VM, Wirtz D, Hanein D, Waterman-Storer CM (2005) Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. J Cell Biol 168:619–631
Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279:509–514
Harlan JE, Yoon HS, Hajduk PJ, Fesik SW (1995) Structural characterization of the interaction between a pleckstrin hamalogy domain and phosphatidylinositol 4,5-bisphosphate. Biochemistry 34:9859–9864
Hartshorne DL, Ito M, Erdodi F (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. J Muscle Res Cell Motil 19:325–341
Hartwig JH, Bokoch GM, Carpenter CL, Janmey PA, Taylor LA, Toker A, Stossel TP (1995) Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 82:643–653
Hirao M, Sato N, Kondo T, Yonemura S, Monden M, Sasaki T, Takai Y, Tsukita Sh, Sa T (1996) Regulation mechanism of ERM (ezrin/redixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. J Cell Biol 135:37–51
Huang TY, DerMardirossian C, Bokoch GM (2006) Cofilin phosphatases and regulation of actin dynamics. Curr Opin Cell Biol 18:26–31
Huttelmaier S, Mayboroda O, Harbeck B, Jarchau T, Jockusch BM, Rudiger M (1998) The interaction of the cell-contact proteins VASP and vinculin is regulated by phosphatidylinositol 4,5-biphosphate. Curr Biol 8:479–488
Ichetovkin I, Grant W, Condeelis J (2002) Cofilin produces newly polymerized actin filaments that are preferred for dendritic nucleation by the Arp2/3 complex. Curr Biol 12:79–84
Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M (2002) Activation of Rac and Cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. Mol Cell Biol 22:6582–6591
Kaibuchi K, Kuroda S, Amano M (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem 68:459–486
Katoh K, Kano Y, Amano M, Onishi H, Kaibuchi K, Fujiwara K (2001) Rho-kinase-mediated contraction of isolated stress fibers. J Cell Biol 153:569–583
Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K (1999) Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase *in vivo*. J Biol Cell 147:1023–1038

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273:245–248

Kłopocka W, Pomorski P (1996) Cytoplasmic calcium transients in *Amoeba proteus* during induction of pinocytotic and non-pinocytotic rosettes. Acta Protozool 35:169–175

Kłopocka W, Rędowicz MJ (2003) Effect of Rho family GTP-binding on *Amoeba proteus*. Protoplasma 220:163–172

Kłopocka W, Rędowicz MJ (2004) Rho/Rho-dependent kinase affects locomotion and actin-myosin II activity of *Amoeba proteus*. Protoplasma 224:113–121

Kłopocka W, Moraczewska J, Rędowicz MJ (2005) Characterisation of the Rac/PAK pathway in *Amoeba proteus*. Protoplasma 224:77–84

Klosses WB, Shattil SJ, Pampori N, Schwartz MA (2001) Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. Nat Cell Biol 3:316–320

Korczyński J, Sobierajska K, Krzemiński P, Wasik A, Wypych D, Pomorski P, Kłopocka W (2011) Is the MLC phosphorylation essential for the recovery from ROCK inhibition in glioma C6 cells? Acta Biochim Pol 58:125–130

Korczyński J, Sobierajska K, Pomorski P, Kłopocka W (in preparation) The role of $\alpha_v\beta_5$ integrins in glioma C6 cells recovery from ROCK inhibition

Kosako H, Yoshida T, Matsumura F, Ishizaki T, Narumiya S, Inagaki M (2000) Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moezin proteins at the cleavage furrow. Oncogene 19:6059–6064

Kozma R, Sarner S, Ahmed S, Lim L (1997) Rho family GTPases and neuronal growth cone remodeling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylocholine and collapse induced by RhoA and lysophosphatidic acid. Mol Cell Biol 17:1201–1211

Liao Z, Seye CI, Weisman GA, Erb L (2007) The P2Y$_2$ nucleotide receptor requires interaction with αv integrins to access and activate G$_{12}$. J Cell Sci 120:1654–1662

Liu J, Liao Z, Camden J, Griffin KD, Garrad RC, Santiago-Pérez LI, Gonzáles FA, Seye CI, Weisman GA, Erb L (2004) Src homology 3 binding sites in the P2Y$_2$ nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. J Biol Chem 279:8212–8218

Luo L (2000) Rho GTPases in neuronal morphogenesis. Nat Rev Neurosci 1:173–180

Lustig KD, Weisman GA, Turner JT, Garrad R, Shiau AK, Erb L (1996) P2U purinoreceptors: cDNA cloning, signal transduction mechanisms and structure-function analysis. In: Chadwick DJ, Goode JA (eds), P2 purinoceptors: localization, function and transduction mechanisms. Wiley, New York, p 193–204.

Mackay DJ, Esch F, Furthmayr H, Hall A (1997) Rho- and rac-dependent assembly of focal adhesion complexes and actin filaments in permeabilized fibroblasts: an essential role for ezrin/radixin/moesin proteins. J Cell Biol 138:927–938

Madaule P, Eda M, Watanabe N, FujisawaK MT, Bito H, Ishizaki T, Narumiya S (1998) Role of citron kinase as a target of the small GTPase Rho in cytokinesis. Nature 394:491–494

Maddox AS, Burridge K (2003) RhoA is required for cortical retraction and rigidity during mitotic cell rounding. J Cell Biol 160:255–265

Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, Narumiya S (1999) Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 285:895–898

Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Sa T, Tsukita Sh (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J Cell Biol 140:647–657

Matsumura F (2005) Regulation of myosin II during cytokinesis in higher eukaryotes. Trends Cell Biol 15:371–377

Matsumura F, Ono S, Yamakita Y, Totsukawa G, Yamashiro S (1998) Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. J Cell Biol 140:119–129

Mermall V, Post PL, Mooseker MS (1998) Unconventional myosin in cell movement, membrane traffic, and signal transduction. Science 279:527–533

Miki H, Miura K, Takenawa T (1996) N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 15:5326–5335

Miki H, Jamaguchi H, Suetsugu S, Takenawa T (2000) IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. Nature 408:732–735

Mouneimne G, Soon L, DesMarais V, Sidani M, Song X, Yip SC, Ghosh M, Eddy R, Backer JM, Condeelis J (2004) Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. J Cell Biol 166:697–708

Moussavi RS, Kelley CA, Adelstein RS (1993) Phosphorylation of vertebrate nonmuscle and smooth muscle myosin heavy chains and light chains. Mol Cell Biochem 127–128:219–227

Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K, Uemura T (2002) Control of actin reorganization by Slingshot, a family of phosphatase that dephosphorylate of ADF/cofilin. Cell 108:233–246

Nobes CD, Hall A (1995) Rho, Rac and Cdc42 regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. Cell 81:53–62

Nobes CD, Hall A (1999) Rho GTPases control polarity, protrusion and adhesion during cell movement. J Cell Biol 144:1235–1244

Ohta Y, Kousaka K, Nagata-Ohashi K, Ohashi K, Muramoto A, Shima Y, Niwa R, Uemura T, Mizuno K (2003) Differential activities, subcellular distribution and tissue expression patterns of three members of Slingshot family phosphatases that dephosphorylate cofilin. Genes Cells 8:811–824

Pak CW, Flynn KC, Bamburg JR (2008) Actin-binding proteins take the reins in growth cones. Nat Rev Neurosci 9:136–147

Peterson TS, Camden JM, Wang Y, Seye CI, Wood WG, Sun GY, Erb L, Petris MJ, Weisman GA (2010) P2Y2 nucleotide receptor-mediated responses in brain cells. Mol Neurobiol 41:356–366

Piekny A, Werner M, Glotzer M (2005) Cytokinesis: welcome to the Rho zone. Trends Cell Biol 15:651–658

Pletjushkina OJ, Rajfur Z, Pomorski P, Oliver TN, Vasiliev JM, Jacobson KA (2001) Induction of cortical oscillations in spreading cells by depolymerization of microtubules. Cell Motil Cytoskeleton 48:235–244

Pollard TD, Blanchoin L, Mulins RD (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu Rev Biophys Biomol Struct 29:545–576

Pomorski P, Watson JM, Haskill S, Jacobson KA (2004) How adhesion, migration, and cytoplasmic calcium transients influence interleukin-1beta mRNA stabilization in human monocytes. Cell Motil Cytoskeleton 57:143–157

Pomorski P, Krzemiński P, Wasik A, Wierzbicka K, Barańska J, Kłopocka W (2007) Actin dynamics in *Amoeba proteus* motility. Protoplasma 231:31–41

Ramachandran C, Patil RV, Combrink K, Sharif NA, Srinivas SP (2011) Rho-Rho kinase pathway in the actomyosin contraction and cell-matrix adhesion in immortalized human trabecular meshwork. Mol Vis 17:1877–1890

Ridley AJ (2001) Rho GTPases and cell migration. J Cell Sci 114:2713–2722

Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399

Ridley AJ, Comoglio PM, Hall A (1995) Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. Mol Cell Biol 15:1110–1122

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. Science 302:1704–1709

Riento K, Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4:446–456

Royal I, Lamarche-Vane N, Lamorte L, Kaibuchi K, Park M (2000) Activation of cdc42, rac PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates cell colony spreading and dissociation. Mol Biol Cell 11:1709–1725

Salhia B, Rutten F, Nakada M, Beaudry C, Berens M, Kwan A, Rutka JT (2005) Inhibition of Rho-kinase affects astrocytoma morphology, motility, and invasion through activation of Rac1. Cancer Res 65:8792–8800

Sanders LC, Matsumura F, Bokoch GM, de Lanerolle P (1999) Inhibition of myosin light chain kinase by p21-activated kinase. Science 283:2083–2085

Schmitz AA, Govek EE, Bottner B, Van Aelst L (2000) Rho GTPases: signaling, migration, and invasion. Exp Cell Res 261:1–12

Sechi AS, Wehland J (2000) The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)$P_2$ influences cytoskeletal protein activity at the plasma membrane. J Cell Sci 113:3685–3695

Shestakova EA, Wyckoff J, Jones J, Singer RH, Condeelis J (1999) Correlation of beta-actin messenger RNA localization with metastatic potential in rat adenocarcinoma cell lines. Cancer Res 59:1202–1205

Sidani M, Wessels D, Mouneimne G, Ghosh M, Goswami S, Sarmiento C, Wang W, Kuhl S, El-Sibai M, Backer JM, Eddy R, Soll D, Condeelis J (2007) Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. J Cell Biol 179:777–791

Sohn RH, Chen J, Koblan KS, Bray PF, Goldschmidt-Clermont PJ (1995) Localization of a binding side for phosphatidylinositol 4,5-biphosphate on human profiling. J Biol Chem 270:21114–21120

Somlyo AP, Somlyo AV (2003) $Ca^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 83:1325–1358

Song X, Chen X, Yamaguchi H, Mouneimne G, Condeelis JS, Eddy RJ (2006) Initiation of cofilin activity in response to EGF is uncoupled from cofilin phosphorylation and dephosphorylation in carcinoma cells. J Cell Sci 119:2871–2881

Stockem W, Kłopocka W (1988) Ameboid movement and related phenomena. Int Rev Cytol 112:137–183

Sun CX, Magalhaes MAO, Glogauer M (2007) Rac1 and Rac2 differentially regulate actin free barbed end formation downstream of the fMLP receptor. J Cell Biol 179:239–245

Suplat-Wypych D, Dygas A, Baranska J (2010) 2′, 3′-O-(4-benzoylbenzoyl)-ATP-mediated calcium signaling in rat glioma C6 cells: role of the P2Y(2) nucleotide receptor. Purinergic Signal 6:317–325

Sutton TA, Mang HE, Atkinson SJ (2001) Rho-kinase regulates myosin activation in MDCK cells during recovery after ATP depletion. Am J Physiol Renal Physiol 281:F810–F818

Takenawa T, Itoh T (2001) Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim Biophys Acta 1533:190–206

Targos B, Pomorski P, Krzemiński P, Barańska J, Rędowicz MJ, Kłopocka W (2006) Effect of Rho-associated kinase inhibition on Actin cytoskeleton structure and calcium response in glioma C6 cells. Acta Biochim Pol 53:825–831

Toker A (2002) Phosphoinositides and signal transduction. Cell Mol Life Sci 59:761–779

Tolias KF, Hartwig JH, Ishihara H, Shibasaki Y, Cantley LC, Carpenter CL (2000) Type Ialpha phosphatidylinositol -4-phasphate 5-kinase mediates Rac-dependent actin assembly. Curr Biol 10:153–156

Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F (2000) Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol 150:797–806

Tsuji T, Ishizaki T, Okamoto M, Higashida C, Kimura K, Furuyashiki T, Arakawa Y, Birge RB, Nakamoto T, Hirai H, Narumiya S (2002) ROCK and mDia1 antagonize in Rho-dependent Rac activation in Swiss 3T3 fibroblasts. J Cell Biol 157:819–830

Van Aelst L, D'Souza-Schorey C (1997) Rho GTPases and signaling network. Genes Dev 11:2295–2322

van Leeuwen FN, Kain HE, Kammen RA, Michiels F, Kranenburg OW, Collard JG (1997) The guanine nucleotide exchange factor Tiam1 affects neuronal morphology: opposing rols for the small GTPases Rac and Rho. J Cell Biol 139:797–807

van Leeuwen FN, van Delft S, Kain HE, van der Kammen RA, Collard JG (1999) Rac regulates phosphorylation of the myosin-II heavy chain, actinomyosin disassembly and cell spreading. Nat Cell Biol 1:242–248

van Rhee AM, Jacobson KA, Garrad R, Weisman GA, Erb L (1998) P2 receptor modeling and identification of ligand binding sites. In: Turner JT, Weisman GA, Fedan JS (eds) P2 nucleotide receptors. Humana Press, Totowa, pp 135–166

van Rheenen J, Song X, van Roosmalen W, Cammer M, Chen X, DesMarais V, Yip S-C, Backer JM, Eddy RJ, Condeelis JS (2007) EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells. J Cell Biol 179:1247–1259

van Rheenen J, Condeelis J, Glogauer M (2009) A common cofilin activity cycle in invasive tumor cells and inflammatory cells. J Cell Sci 122:305–311

Wang M, Kong Q, Gonzalez FA, Sun G, Erb L, Seye C, Weisman GA (2005) $P2Y_2$ nucleotide receptor interaction with $\alpha_v$ integrin mediates astrocyte migration. J Neurochem 95:630–640

Watanabe N, Kato T, Fujita A, Ishizaki T, Narumiya S (1999) Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat Cell Biol 1:136–143

Welch MD, Mullins RD (2000) Cellular control of actin nucleation. Annu Rev Cell Dev Biol 18:247–288

Wong WT, Faulkner-Jones B, Sanes JR, Wong RO (2000) Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. J Neurosci 20:5024–5036

Worthylake RA, Lemoine S, Watson JM, Burridge K (2001) RhoA is required for monocyte tail retraction during transendothelial migration. J Cell Biol 154:147–160

Yamashiro S, Totsukawa G, Yamakita Y, Sasaki Y, Madaule P, Ishizaki T, Narumiya S, Matsumura F (2003) Citron kinase, a Rho-dependent kinase, induces diphosphorylation of regulatory light chain of Myosin II. Mol Biol Cell 14:1745–1756

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K (1998) Cofilin phosphorylation by LIM-kinase1 and its role in Rac-mediated actin reorganization. Nature 393:809–812