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Rho GTPase regulation by miRNAs and covalent modifications

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Abstract

To date, most studies of Rho GTPase regulation have focused on the classic GTPase cycle – GTP binding and hydrolysis – controlled by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GDP-dissociation inhibitors (GDIs). Recent investigations have unveiled important additional regulatory mechanisms: microRNA (miRNA) regulating post-transcriptional processing of Rho GTPase-encoding mRNAs; palmitoylation and nuclear targeting affecting intracellular distribution; post-translational phosphorylation, transglutamination and AMPylation impacting Rho GTPase signaling; and ubiquitination controlling Rho GTPase protein stability and turnover. These modes of regulation add to the complexity of the Rho GTPase signaling network and allow precise spatiotemporal control of individual Rho GTPases. This review will discuss these 'unconventional' modes of regulation and their contribution to cellular function, focusing on post-transcriptional and post-translational events beyond the classic GTPase cycle regulatory model.

Keywords

small GTPases; microRNAs; ubiquitination; phosphorylation; transglutamination

An overview of the Rho GTPase regulatory cycle

The Rho GTPases, which belong to the Ras superfamily of 20–30 kDa GTP-binding proteins, include at least 20 members in higher eukaryotes that can be subdivided into six groups: Rho subfamily (RhoA, RhoB, RhoC); Rac subfamily (Rac1, Rac2, Rac3, RhoG); CDC42 subfamily (CDC42, Wrch1, TC10, Chp, TCL); Rnd subfamily (Rnd1, Rnd2, Rnd3); Rho BTB subfamily (RhoBTB1, RhoBTB2, RhoBTB3); and Miro subfamily (Miro1, Miro2) [1,2]. These proteins function in multiple cell processes including gene expression, cytoskeletal dynamics, survival, cell division, cell adhesion, polarity and vesicle trafficking [1–4]. Dysfunctional Rho GTPase regulated signaling underlies multiple forms of cancer, neurological abnormalities, immunological disorders and several other diseases [5–10]. While a subset of Rho GTPases are constitutively active, the majority act as molecular switches, cycling between the active, GTP-bound form and the inactive, GDP-bound form. Their activities can be influenced by multiple types and levels of spatiotemporal regulation

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that puts them in the context of a vast intracellular signaling network [11–17]. Upon activation, each Rho GTPase may interact with several effector targets leading to physiologic responses.

Rho GTPases can be activated by intrinsic or extrinsic cues, setting off a signaling cascade [1,3,15]. In response to stimulatory signals, individual Rho GTPase activities are controlled by the GTP/GDP ratio and subcellular distribution in the cell through the joint work of multiple regulatory molecules: GEFs, which activate Rho GTPases by promoting GDP-to-GTP exchange; GAPs, which inactivate the GTPases by enhancing intrinsic GTP hydrolysis activity; and GDIs, which bind prenylated GDP-bound Rho proteins, allowing translocation of Rho GTPases between membranes and cytosol [11–14,17] and protecting Rho GTPases from degradation [18]. This molecular switch regulatory mechanism forms the classic ‘GTPase cycle’ model (GTP-binding/GTP-hydrolysis) that has been the subject of extensive reviews previously [15–17].

Adding to the complexity of the GTPase cycle, recent studies have revealed a few ‘unconventional’ mechanisms for the regulation of Rho GTPase signaling activities. These include post-transcriptional regulation by microRNAs (miRNAs), intracellular distribution by lipid and nuclear translocation signals, post-translational modifications via phosphorylation, transglutamination and AMPylation, and protein stability controlled by ubiquitination (Figure 1). In this review, we discuss these additional modes of Rho GTPase regulation, focusing on post-transcriptional regulation and post-translational covalent modifications. We present evidence that these mechanisms, combined with the GTPase cycle, are important for maintaining physiological levels of Rho GTPase activity in cells.

miRNA regulation of Rho GTPase expression

To date, more than 1500 miRNAs have been identified in human cells [19]. These short, non-coding RNA molecules play critical roles in diverse physiological, developmental and pathological processes by controlling gene expression post-transcriptionally. In conjunction with Argonaute protein, miRNAs can silence target genes by either suppressing translation or degrading mRNA – in the former case, by partially complementing the 3′ untranslated region (3′ UTR) of cognate mRNAs to suppress protein synthesis [20–22]. By regulating one or more mRNA targets, individual miRNAs can direct a developmental switch or tissue-specific gene expression [20–22]. Through targeting and regulating Rho GTPase-encoding mRNAs, a number of miRNAs have been implicated in the regulation of gene expression of Rho GTPases (Table 1), thereby influencing pathophysiologic functions such as cardiac function, neuronal differentiation and tumorigenesis.

Rho GTPases, RhoA and Cdc42 in particular, figure prominently in cardiac development and hypertrophy, and recent studies suggest that miRNAs contribute to their regulation. For example, miR-133 is down-regulated in mouse models and human patients with cardiac hypertrophy, and inhibits cardiac hypertrophy by targeting RhoA and Cdc42, which control cytoskeletal and myofibrillar rearrangements during hypertrophy, suggesting its potential therapeutic application in heart disease [23]. Similarly, miR-1, which targets Cdc42, is negatively regulated by homeobox transcription factor Tinman in the fly and its mammalian homologue Nkx2-5 in the mouse, both of which are involved in heart development and function [24]. miRNA targeting of Rho GTPases also regulates neuronal differentiation. miR-124, which is expressed in developing and adult neurons, regulates axon growth by suppressing Cdc42 and Rac1 expression [25].

Alterations in Rho GTPase gene expression levels, rather than constitutive mutations, often are associated with tumorigenesis and cancer progression. The cause of such an expression change may be attributed, at least in part, to regulation by miRNAs impacting abnormal

signaling activity and function. Indeed, miRNAs are involved in many types of cancer, where they may regulate key processes of tumorigenesis and tumor progression, including metastasis, apoptosis, proliferation, and angiogenesis, by targeting oncogenes or tumor suppressor genes [26], including the Rho GTPases. The levels of miR-31 are inversely correlated with metastasis activity in human breast tumors, and miR-31 suppresses metastasis *in vivo* by coordinating repression of a cohort of metastasis-promoting genes, including RhoA; expression of exogenous RhoA partially reverses the metastasis defects induced by miR-31 overexpression [27,28]. On the other hand, miR-155, which is regulated by the transforming growth factor beta/Smad pathway, targets RhoA mRNA and contributes to epithelial-mesenchymal transition (EMT) and tight junction dissolution, as well as cell migration and invasion [29]. In addition, cell migration and invasion are positively regulated by the pro-metastatic Rho GTPase RhoC, whose expression is controlled indirectly through miR-10b [30]. In this case, the transcription factor Twist induces miR-10b, which inhibits translation of homeobox D10 mRNA to cause an increase in expression of RhoC [30].

In addition to suppressing metastasis of cancer cells, miRNAs can also negatively regulate proliferation, cell cycle and survival by targeting Rho GTPases. miR-137, which is aberrantly down-regulated in cancers [31], inhibits proliferation and invasion and induces G1 cell-cycle arrest in colorectal and gastric cancer cells. It does this by targeting Cdc42 mRNA at nucleotides (nt) 792 to 798 in the 3' UTR – a region that is highly conserved [32,33]. Furthermore, miR-137 effectively inhibits Pak-MLC and ERK signaling in colorectal cancer cell lines, likely through suppression of Cdc42 [33]. Sequence analysis indicates that the 3' UTRs of RhoA (nt 1844–1852) and Cdc42 (nt 1382–1396) encode miR-185 target-matching sequences and are highly conserved across species [34]. miR-185 can inhibit the expression of RhoA and Cdc42 mRNAs through these matched 3' UTRs to inhibit proliferation and invasion, and induce G1 cell cycle arrest and apoptosis, mimicking the effects of knocking down RhoA and/or Cdc42 expression in colorectal cancer cells [34]. miR-29 family members (i.e. miR-29a, -29b and -29c) are capable of upregulating p53 to induce apoptosis; this is mediated by directly suppressing p85-alpha and Cdc42, which are negative regulators of p53 [35].

In some cases, miRNAs targeting Rho GTPases may positively regulate tumor cell functions. For instance, miR-21, an oncogenic miRNA that is highly expressed in multiple tumor types [36,37], positively regulates cell proliferation, migration, invasion, metastasis and survival – at least in part by targeting the tumor suppressor RhoB [38,39].

Subcellular distribution of Rho GTPases

Subcellular localization of Rho GTPases is an important mechanism for regulating their spatially unique functions. Many Rho proteins, for instance, cycle from the cytosol to plasma membranes, guided by a lipid isoprenylation moiety at a conserved cysteine residue at the carboxy-terminal CAAX motif to provide a membrane anchor [41,42] (Figure 2). Working in the opposite direction, RhoGDIs bind to and negatively regulate Rho GTPases by extracting GTP-bound GTPases from the plasma membrane, and sequestering them in the cytosol in an inactive conformation [13]. A recent study has unveiled that Rac1 can incorporate palmitate at cysteine-178 after prenylation, which is regulated by the triproline-rich motif. This posttranslational modification targets Rac1 for stabilization at actin cytoskeleton-linked, ordered membrane regions [43]. On the other hand, isoprenylation and palmitoylation of RhoB seem to have different consequences – inducing endo-lysosomal localization and rapid degradation [44]. Palmitoylation might also be a regulatory mechanism for other Rho GTPases – such as RhoU [45] and RhoV [46] – associated with subcellular localization. During cell signaling, coronin1A can induce Rac1 translocation

from the cytosol to membranes and activation via formation of an F-actin-dependent heteromolecular complex with ArhGEF7, Pak1, and RhoGDI [47].

Several Rho GTPases contain a polybasic region (PBR) – a series of adjacent lysine or arginine residues that often immediately precede the C-terminal CAAX sequence [48]. The PBR of Rac1, but not RhoA, has significant nuclear localization signal (NLS) activity (Figure 2). Nuclear accumulation of both Rac1 and SmgGDS is enhanced by Rac1 activation and diminished by mutation of the Rac1 PBR [49]. SmgGDS, the only known RhoGEF of the armadillo (ARM) family of proteins, shuttles between the nucleus and cytoplasm, similar to other ARM proteins [49]. Moreover, nuclear import of Rac1 is mediated by direct interaction with Karyopherin α 2, a member of the alpha importin family of karyopherins that are involved in the first step of nuclear import – docking of proteins to the nuclear envelope [50,51]. Although this interaction is independent of GTP loading of Rac1, the second step of nuclear import – translocation across the nuclear envelope – is dependent on Rac1 activation [51].

Endogenous RhoA may also have a presence in the nucleus, along with upstream signaling partners RhoA-GAP DLC1 and p190 RhoGAP and downstream effectors ROCK II/ROKa and LIMK [52–54]. Rho GDI1 interacts strongly with the PBR of RhoA, thereby keeping RhoA sequestered in the cytosol and away from membranes where it is active. When Rho GDI1 is depleted or silenced, the fraction of nuclear RhoA increases significantly, despite an overall reduction in RhoA protein [55].

The functional consequences of nuclear localization of Rac1 and RhoA – as well as other Rho GTPases – remain largely unknown. They may fulfill specific nuclear functions such as serving as co-transcriptional factors to regulate the cell cycle. Alternatively, sequestration of Rho GTPases in the nucleus could be a means to terminate cytosolic functions through ubiquitylation-mediated degradation.

Transglutamination, Phosphorylation and AMPylation of Rho GTPases

Rho GTPases are the preferred targets of various bacterial cytotoxins, including CNF1 from *Escherichia coli* and dermonecrotizing toxin (DNT) from *Bordetella* species. Cytotoxins commonly inactivate or constitutively activate Rho GTPases by covalently crosslinking bulky molecules (e.g. a phosphate group, amidocyanogen or AMP) to a crucial residue in the switch I or II region of the Rho GTPase structures essential for signaling functions. In addition, ADP-ribosylation and glucosylation represent two other types of covalent modification of Rho GTPases, both of which may lead to inactivation of Rho GTPase activities. These important regulatory mechanisms have been reviewed elsewhere recently [56]. Interestingly, a very recent study shows that Rac1, and particularly GTP-bound Rac1, can be SUMOylated in the polybasic region *in vitro* and *in vivo*, which is not required for its localization but is needed for optimal GTP loading and activation [57].

Transglutamination

The carboxamide nitrogen of the switch II region stabilizes the transition state during GTP hydrolysis, and is thus essential to the intrinsic and GAP-stimulated GTPase activity of small GTPases. Transglutamination permanently activates Rho GTPase proteins by targeting this essential switch II glutamine residue (Figure 3a) [58,59]. In addition, CNF1 catalyzes transglutamination of the conserved glutamine-63 of RhoA (glutamine-61 of Rac1 and Cdc42), which abolishes the intrinsic and GAP-stimulated GTPase activity – leading to permanent activation and inducing reorganization of actin cytoskeleton [58,59]. Upon activation by retinoic acid (RA), the tissue transglutaminase II (TGase) constitutively activates RhoA by targeting glutamine-63, leading to increased binding to a downstream

target, RhoA-associated kinase-2 (ROCK-2) [60]. Similarly, RA increases expression of and activates TGase in SH-SY5Y cells, which leads to transamidation and, thus, activation of RhoA – thereby promoting formation of stress fibers and focal adhesion complexes, and inducing neuronal differentiation. Transamidated RhoA also promotes activation of ERK1/2 and p38 γ MAP kinase [61]. In addition, serotonin-induced transamidation (serotonylation) of RhoA in hypoxic rats and patients with idiopathic pulmonary hypertension (iPH) could be involved in pulmonary artery remodeling and hypertension [62,63].

Phosphorylation

Another way to covalently modify Rho GTPases is through protein kinase-mediated phosphorylation events. Several kinases, such as PKA, PKG, Src or Akt, can directly phosphorylate RhoA, Cdc42 and/or Rac1 [64–67] (Figure 3b). RhoA is a substrate for cAMP-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G (PKG). Both phosphorylate RhoA at serine-188, which does not affect its intrinsic biochemical properties, but does trigger tight association of its GTP-bound form with RhoGDI, and extraction of RhoA from membranes [64,65,68]. Nerve growth factor (NGF) triggers phosphorylation of serine-188 on RhoA by PKA – blocking the ability of RhoA to associate with Rho-associated kinase (ROK), but not affecting its ability to interact with other targets (e.g. rhotekin, mDia-1 and PKN) [69], suggesting that phosphorylation of RhoA by PKA can differentially affect RhoA binding to its effectors [69]. In addition, the phosphorylation of RhoA in vascular smooth muscle cells has been implicated in the regulation of vascular homeostasis, and several feedback regulations have been described [70–72]. Based on a hypertensive knockout mouse lacking the Rho/Rac activator Vav2, a new signaling pathway involving Vav2, the GTPase Rac1 and the serine/threonine kinase Pak, has been discovered to contribute to nitric oxide – triggered blood vessel relaxation and normotensia [72]. A separate study showed that phosphorylation of tyrosine-64 on Cdc42 by Src tyrosine kinase increased its association with GDIs [66]. On the other hand, phosphorylation of serine-71 on Rac1 by serine/threonine kinase Akt seems to have different effects – inhibiting the GTP-binding activity of Rac1 with no significant change in GTPase activity [67]. Phosphorylation might also be a regulatory mechanism for other Rho GTPases – such as Rnd3/RhoE [73], RhoB [74] and RhoH [75]. Rnd3, for example, can be phosphorylated by ROCK I, and this phosphorylation increases Rnd3 stability and leads to subsequent cytosol localization, correlating with its ability to induce actin stress fibre disruption and inhibit Ras-induced cell transformation [73]. Compared with conventional regulation by GAPs, phosphorylation of most Rho GTPases may maintain a reservoir of GTP-loaded Rho GTPases that can be mobilized in the absence of activation by GEFs, or are inaccessible to activation by GEFs [76].

AMPylation

AMPylation represents a newly discovered posttranslational modification used to stably modify RhoGTPases with AMP. *Yersinia* YopT and Fic domains of prokaryotic immunoglobulin-binding protein A (IbpA) are distinct protein modification enzymes. YopT is a cysteine protease that cleaves and inactivates Rho GTPases [77], while IbpA induces cytotoxicity in infected cells by catalyzing an adenosine monophosphate (AMP) modification on threonine residues of protein substrates. Transient transfection of HeLa cells with EGFP-YopT or IbpA led to drastic cytoskeletal collapse by targeting the host Rho GTPases [77–79]. The IbpA-induced reaction depends on the presence of a conserved histidine in the Fic domain's core motif HPFxxGNR, and covalent attachment of AMP by a phosphodiester bond, which may be reversibly removed by phosphodiesterases [80]. The resulting AMPylation prevents Rho GTPase interaction with downstream effectors, thereby inhibiting actin assembly and inducing cell rounding [78,79] (Figure 3c). Huntingtin yeast-interacting protein E (HYPE), the only human protein containing a Fic domain, is also

capable of adding AMP to RhoA, Rac1 and Cdc42 and catalyzing the modification of similarly sized proteins *in vitro*; mutant HypE (H295A) cannot do this. Although prokaryotic and HYPE Fic motifs are highly similar, ectopic expression of HYPE in HeLa cells did not induce a cell-rounding phenotype; perhaps because HYPE expression is tightly controlled in mammalian cells and/or its activity is compartmentalized [79]. Understanding the diverse mechanisms used by bacterial effectors and toxins to regulate host GTPase activity will help assess the pathogenicity and toxicity of bacterial pathogens, and prevent the diseases that they cause. Bacterial protein toxins that modify and regulate host Rho GTPases are the subject of an excellent recent review [81].

Ubiquitylation-based regulation of Rho GTPases

Ubiquitylation, the covalent attachment of ubiquitin or polyubiquitin to eukaryotic proteins, regulates a broad range of critical cellular functions overlapping with that of Rho GTPases such as cell survival, cytoskeletal organization, cell-cycle progression, vesicle trafficking and cell migration. Covalent attachment of the 8-kDa ubiquitin polypeptide to lysine residues on the target depends on a cascade of transfer reactions between ubiquitin-carrier proteins. Polyubiquitin chains are formed when additional ubiquitin molecules are attached to one of the seven lysine residues of the previously cross-linked ubiquitin molecule [82]. At the protein level, members of the Rho GTPase family, Rac1, RhoA and Cdc42 in particular, are subject to regulation by the ubiquitin-proteasome system (UPS) in a balancing act with constitutive activation [83,84] (Figure 4).

The first evidence of Rac GTPase regulation by ubiquitylation came from Rac1-induced reactive oxygen species production on the turnover of Rac1 itself in human aortic endothelial cells [85]. Rac1 turnover is indirectly regulated by UPS in a redox-sensitive fashion [85]. Inhibition of NADPH oxidase activity is connected to a proteasome-dependent increase in active Rac1 expression, but not inactive Rac1 – consistent with the effects of proteasome inhibitors [85]. Inhibition of Rac1 by UPS-mediated degradation also occurs at EMT onset – during the early stages of epithelial cell scattering [86]. Rac1 protein levels may also be controlled by Caveolin 1 (Cav1) regulation of UPS-mediated degradation of activated Rac1, in an adhesion-dependent fashion – further supporting the notion that Rac1 signaling during EMT is inhibited by UPS-mediated degradation, in addition to or instead of inactivation by Rho GAPs [87]. Moreover, Cav1 deletion leads to an increase in non- and mono-ubiquitylated Rac1, suggesting that Cav1 selectively regulates degradation of poly-ubiquitylated activated Rac1 [87,88]. Mutational analysis of all lysine residues in Rac1 revealed that ubiquitin chains are preferentially cross-linked to lysine-147, a solvent-accessible residue with a similar conformation in Rac1b, an alternative splice form of Rac1 [89]. The HECT-domain-containing E3-ubiquitin-ligase tumor suppressor HACE1 preferentially binds to GTP-bound Rac1 for ubiquitylation [90]. HACE1 expression increases the ubiquitylation of Rac1, while RNAi-mediated depletion of HACE1 blocks the ubiquitylation of active Rac1 and increases levels of GTP-bound Rac1 [90].

RhoA, constitutively activated by cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli*, is also subject to UPS-mediated degradation [84,91]. The E3 ligase Smurf1, which regulates Smad protein stability, directly targets RhoA for UPS-mediated degradation – thereby regulating epithelial cell morphology [92], cell polarity [93,94], protrusive formation [93,95], neurite outgrowth [96] and tumor-cell migration and invasion (in the last case by inhibiting Rho kinase [ROCK] activity and myosin light chain 2 phosphorylation) [97]. Ubiquitin chains are primarily cross-linked to two lysine residues located at the N-terminus of RhoA in this process [92], whereas the C2 domain of Smurf1 is necessary and sufficient for binding RhoA and, therefore, targeting it for ubiquitination [95]. Interestingly, PKC ζ , an effector of the Cdc42/Rac1-PAR6 polarity complex, binds to and colocalizes with

Smurf1 at membrane protrusions, where it controls local downregulation of RhoA signaling to promote cell motility [93]. Cullin-3 also shows E3 ligase activity for RhoA, but does not bear any resemblance to Smurf1 [98]. RhoA is ubiquitinated when RhoA-binding BTB domain adaptors (BACURDs) form complexes with ubiquitin and Cullin-3 ligase [98]. Furthermore, Cullin-3 specifically targets GDP-bound RhoA for ubiquitination – not GTP-bound RhoA, RhoB, RhoC, Cdc42 or Rac1 [98]. Similar to suppression of Smurf1, loss of Cullin-3 and/or BACURD leads to increased RhoA protein levels, and dramatically promotes actin stress fiber formation [98]. As with RhoA and Rac1, CNF1-activated Cdc42 is also subject to UPS-mediated degradation, although the mechanism by which it is targeted for ubiquitylation is not well understood [84].

Just as ubiquitination of Rho GTPases is critical for proper regulation of numerous cell processes, removal of ubiquitin by deubiquitinating enzymes (DUBs) may also be an important step in Rho GTPase stability control, and this aspect of our understanding has only begun to emerge. For example, Cav1 selectively regulates mono-ubiquitylated Rac1, which can be either de-ubiquitylated by members of the ubiquitin-specific protease (USP) family or poly-ubiquitylated, followed by proteasomal degradation [87,88]. While de-ubiquitination is not well understood, DUBs play a central role. Loss of USP17, an immediate-early gene that belongs to a subfamily of cytokine-inducible DUBs, blocks normal cytoskeletal rearrangements and chemokine-induced membrane localization of RhoA, Rac1 and Cdc42 – perhaps mediated by RCE1 and/or other targets [99].

Concluding remarks

It has been over two decades since three major classes of regulators for the Rho family GTPases, RhoGEFs, RhoGAPs and RhoGDIs, were identified. This led to a widely accepted GTPase cycle model for Rho GTPase regulation in which most Rho family members act as molecular switches, similar to what has been proposed for other Ras-superfamily small GTPases. Recent advances in this field have revealed several additional regulatory mechanisms, discussed in this review, that yield a broader array of controls for fine-tuning the activity and function of individual Rho GTPases. In addition to what is presented here, epigenetic modification of Rho GTPase genes may also play a role (Figure 1) as suggested by recent studies of RhoB and RhoE regulation by histone deacetylation [100,101]. It is very likely that future research will reveal similar regulatory mechanisms, via miRNA or covalent modifications, for the classical Rho regulators (i.e. GEF, GAP and GDI); to this end, extensive studies have pointed to phosphorylation as one of the major events regulating Rho GAP and Rho GEF activities [102,103]. These possible regulatory mechanisms will each need to be analyzed in various physiological, pathological and signaling contexts because each mode of regulation could be context-dependent, as individual Rho GTPases may function differently in each cell type [3,4]. For subcellular localization of Rho GTPases, the exact mechanism and functional consequences of nuclear translocation must be clearly defined. The complexity of Rho GTPase regulation in time and intracellular space will likely continue to grow, particularly in the network of small GTPase crosstalk [104]. Such added sophistication of Rho GTPase regulation mechanism allows cells to strictly coordinate the activities of multiple small GTPases and associated pathways in controlling diverse cell behaviors.

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Glossary

RhoA	Ras Homolog gene family, member A
Cdc42	Cell Division Cycle 42
Rac1	Ras-related C3 botulinum toxin substrate 1
GEFs	Guanine nucleotide Exchange Factors
GAPs	GTPase Activating Proteins
GDI s	Guaninenucleotide-Dissociation Inhibitors
UPS	Ubiquitin-Proteasome System
CNF1	Cytotoxic Necrotizing Factor 1
HACE1	HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase
DNT	Dermonecrotizing Toxin
ROCK-2	RhoA-associated kinase-2
HYPE	Huntingtin Yeast-interacting Protein E
Coro1A	Coronin1A
NLS	Nuclear Localization Signal
EMT	Epithelial-Mesenchymal Transition
PKA	cAMP-dependent Protein Kinase A
PKG	cGMP-dependent Protein Kinase G
PDEs	Phosphodiesterases
KPNA2	Karyopherin α 2

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Figure 1.

An overview of Rho GTPase regulatory mechanisms. GEFs, GAPs and GDIs represent the classic regulatory components controlling the GTP-binding/GTP-hydrolysis GTPase cycle and signaling activities of Rho GTPases. GEFs activate Rho GTPases by catalyzing the exchange of bound GDP for GTP while GAPs stimulate the hydrolysis of GTP by active Rho-GTP species thereby inactivating Rho GTPases. RhoGDIs can bind to prenylated GDP-bound Rho proteins and allow translocation of Rho-GDP between membranes and the cytosol. 'Unconventional' regulatory modes are also involved in controlling Rho GTPase activities and cellular functions. First, epigenetic modifications of chromosomal DNAs and miRNAs of Rho GTPases can regulate the expression of Rho GTPases, at pre- and post-transcriptional levels. Second, palmitoylation, prenylation and nuclear localization signals (NLS) of a subset of Rho GTPases can target them to proper intracellular compartments to execute cell functions. Third, post-translational covalent modifications, including phosphorylation, transglutamination, AMPylation and SUMOylation, can induce constitutive activation or inactivation of Rho GTPases or change their activities. Fourth, Rho GTPase activation could be counterbalanced by a loss of active Rho GTPases at the protein level through the ubiquitin – proteasome system (UPS). Combined, these regulatory

mechanisms contribute to the spatiotemporal modulation of the signaling strength of Rho GTPases in diverse cellular processes, impacting on actin and microtubule dynamics, cell adhesion, cell cycle progression, cell survival, gene expression, polarity establishment and membrane transport.

Figure 2.

Regulation of subcellular localization of Rac1 GTPase. Rac1 can incorporate palmitate at cysteine 178 after prenylation, which is regulated by the C-terminal triproline-rich motif. This posttranslational modification stabilizes Rac1 at actin-cytoskeleton-linked ordered membrane regions. The C-terminal polybasic region (PBR) of Rac1 has NLS activity. Nuclear import of Rac1 is mediated by a direct interaction with Karyopherin $\alpha 2$, an importin that transfers cargo molecules to the nucleus. GDIs bind to the isoprenylated Rho GTPases and negatively regulate Rho GTPases by extracting GTP-bound GTPases from the plasma membrane, and sequestering them in the cytosol in an inactive conformation.

Figure 3.

Transglutamination, phosphorylation and AMPylation of Rho GTPases to modulate signaling strengths. **a.** CNF1 catalyzes the deamidation of Glu63 of RhoA and Glu61 of Rac1 and Cdc42 into a glutamic acid, which blocks the GTP-hydrolytic activity of the Rho proteins stimulated by GAPs. Such a modification leads to constitutive activation of the Rho GTPases. **b.** Several cellular kinases, including PKA, PKG, Src or Akt, can phosphorylate RhoA at Ser188 or Cdc42 at Tyr64, respectively, and this covalent modification may trigger a tight association of Rho-GDI with RhoA in its GTP-bound form. **c.** Fic domains of IbpA and HYPE (Huntingtin yeast-interacting protein E) induce cytotoxicity by catalyzing an adenosine monophosphate (AMP) modification on threonine residues of RhoA, Rac1 and Cdc42. The resulting AMPylation attachment prevents the interaction of these Rho GTPases with downstream effectors. The covalent attachment of AMP by a phosphodiester bond to the Rho GTPases is predicted to be reversibly catalyzed by phosphodiesterases (PDEs).

Figure 4.

Regulation of Rho GTPase protein stability by ubiquitylation. RhoA, Rac1 and Cdc42 can be subject to ubiquitylation-mediated proteasomal degradation. Smurf1, a HECT domain E3 ubiquitin ligase, is capable of regulating cell polarity and protrusion formation by specifically targeting GTP-bound RhoA for ubiquitylation and subsequent proteasomal degradation under the control of PKC ζ , an effector of the Cdc42/Rac1-PAR6 polarity complex. Cullin-3 specifically targets GDP-bound RhoA for ubiquitylation through the RhoA binding BTB domain adaptors, termed BACURDs, to induce membrane protrusion, reduce actin stress fiber formation and inhibit cell motility. The tumor suppressor HACE1, a HECT-domain containing E3 ubiquitin-ligase, binds preferentially to GTP-bound Rac1 to

introduce polyubiquitylation. In addition, the membrane-associated adapter protein caveolin-1 (Cav1) controls Rac1 protein turnover by regulating the ubiquitylation of activated Rac1 in an adhesion-dependent fashion. Mono-ubiquitylated Rac1 can be subject to reversible modulation by either poly-ubiquitylation for proteasomal degradation or de-ubiquitylation by members of the USP family. Similar to RhoA and Rac1, Cdc42 is susceptible to CNF1-mediated degradation, but the mechanism by which Cdc42 is targeted for ubiquitylation remains to be defined.

Table 1

Posttranscriptional regulation of Rho GTPases by miRNAs

miRNA	Rho GTPase (Target)	miRNA expression change in cancer	Functional contribution	Reference
miR-31	RhoA	Glioblastoma↓, breast cancer↓	Metastasis	[27,28]
miR133		ND [/]	Cardiac hypertrophy	[23]
miR-155		Colon cancer↑, breast cancer↑	EMT	[29]
miR-185		Colorectal cancer↑	Proliferation	[34]
miR-1	Cdc42	ND	Heart function	[24]
miR-29		Breast cancer↓, CLL↓, lung cancer↓	Apoptosis	[35]
miR-124		ND	Neurite outgrowth	[25]
miR-133		ND	Cardiac hypertrophy	[23]
miR-137		Colorectal cancer↓, Glioblastoma multiforme↓	Proliferation, invasion	[32,33]
miR-185		Colorectal cancer↑	Proliferation	[34]
miR-124	Rac1	ND	Neurite outgrowth	[25]
miR-21	RhoB	Colorectal cancer↑, breast cancer↑	Proliferation, metastasis	[38,39]
miR-200b	RhoE	ND	Cell cycle	[40]

[/]ND: Not determined in cancer in relationship with Rho GTPases.