Regulating Rho GTPases and their regulators

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Abstract | Rho GTPases regulate cytoskeletal and cell adhesion dynamics and thereby coordinate a wide range of cellular processes, including cell migration, cell polarity and cell cycle progression. Most Rho GTPases cycle between a GTP-bound active conformation and a GDP-bound inactive conformation to regulate their ability to activate effector proteins and to elicit cellular responses. However, it has become apparent that Rho GTPases are regulated by post-translational modifications and the formation of specific protein complexes, in addition to GTP–GDP cycling. The canonical regulators of Rho GTPases — guanine nucleotide exchange factors, GTPase-activating proteins and guanine nucleotide dissociation inhibitors — are regulated similarly, creating a complex network of interactions to determine the precise spatiotemporal activation of Rho GTPases.

Integrins
A family of transmembrane cell adhesion receptors that bind to extracellular matrix ligands and other cell adhesion receptors, and interact intracellularly with cytoskeletal proteins.

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The Rho GTPase family forms part of the Ras superfamily and consists, in humans, of 20 members, which can be classified into eight subgroups[1] (TABLE 1). Rho GTPases are best known for their roles in regulating cytoskeletal rearrangements, cell motility, cell polarity, axon guidance, vesicle trafficking and the cell cycle[2]. Alterations in Rho GTPase signalling contribute to malignant transformation, neurological abnormalities and immunological diseases.

Most Rho GTPases cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. This cycling is regulated by three types of protein[3] (FIG. 1). Guanine nucleotide exchange factors (GEFs) catalyse the exchange of GDP for GTP, thereby activating the GTPase[4], whereas GTPase-activating proteins (GAPs) increase the intrinsic GTP hydrolysis rate of the GTPase, thereby inactivating it. Guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of some GTPases in the cytosol and prevent them from localizing to membranes or being activated by GEFs. Rho GTPases are rapidly activated by various cell-surface receptors, including integrins, cadherins, cytokine receptors, Tyr kinase receptors and G protein-coupled receptors[4]. In the GTP-bound active conformation, Rho GTPases interact with a range of effector proteins, including kinases, actin regulators and adaptor proteins, leading to changes in cell behaviour. A single Rho GTPase can activate a range of cellular responses, depending on the stimulus and cell type. The spatiotemporal regulation of each Rho GTPase is therefore important to determine the outcome of its activity.

Mechanisms other than cycling also affect Rho GTPase signalling. 'Atypical' GTPases, for example, are not generally regulated by GTP–GDP cycling and therefore do not require GEFs and GAPs. Instead, they are constitutively GTP-bound, because they either possess high intrinsic nucleotide exchange activity or have substitutions in their GTPase domain that prevent GTPase activity. Signalling by these GTPases is controlled by other mechanisms, including post-translational modifications (PTMs). Rho GTPases can also be regulated at the level of gene expression or post-transcriptionally (for example, by microRNAs); a discussion of these types of regulation, however, is beyond the scope of this article (see REFS 5,6 for reviews). Research into the PTMs of Rho GTPases and their regulators by phosphorylation, ubiquitylation and sumoylation has increased substantially over the past few years and has consequently enhanced our appreciation of the important role these modifications have, in combination with GTP–GDP cycling and interactions with other proteins, in controlling Rho GTPase activity. In this Review, we discuss how PTMs impart specificity of signalling by both classical and atypical Rho GTPases, influencing not only the Rho GTPases themselves but also their GEF, GAP and GDI regulators.

Regulation of Rho GTPases

Rho GTPases are regulated by a wide range of PTMs, which function together to ensure that these proteins are activated in an appropriate spatiotemporal fashion. Lipid modifications have a crucial role in determining the subcellular localization of Rho GTPases,

Table 1 | **Post-translational modifications of Rho GTPases***

Rho GTPase	Phosphorylation	Ubiquitylation	Sumoylation
CDC42	• SRC → Y64 — enhancement of RhoGDI interaction[137] • PKA → S185 — translocation to the cytosol by increasing interaction with RhoGDI[25]	—	—
RhoQ	• CDK5 → T197 — maintains RhoQ in lipid raft compartments, thereby disrupting cortical actin[138] • ‡aPKC → T197 — might regulate RhoQ role in coordinating actin remodelling during the fusion/docking of GLUT4 vesicles in response to insulin stimulation[138]	—	—
RAC1	• ERK → T108 — targets RAC1 for translocation to the nucleus[32] • FAK → Y64 — negative regulator of RAC1 activity and cell spreading[30] • SRC → Y64 — negative regulator of RAC1 activity and cell spreading[30] • AKT → S71 — inhibits GTP binding and decreases RAC1 activity[31]	• XIAP and cIAP1 → K147 — polyubiquitylation and proteasomal degradation, regulates plasticity of cell migration[59] • HACE1 → K147 — preferentially targets GTP-bound, active RAC1 for degradation, to control RAC1 activity[61,62,64] • §SCF^{FBXL19} → K166 — proteasomal degradation in a AKT-dependent manner[65]	‖PIAS3 → K183, K184, K186, K188 — results in increased GTP binding and RAC1 activation. Does not seem to be important in RAC1 localization[67]
RAC3	—	SCF^{FBXL19} → K166 — targets RAC3 for proteasomal degradation and results in E-cadherin downregulation[66]	—
RhoA	• ¶PKA → S188 — translocation to the cytosol by increasing interaction with RhoGDI[21–23] • Decreases binding and inhibits activity of ROCK effector[26] • Protects GTP-bound RhoA from proteasome-mediated degradation • PKG → S188 — translocation to the cytosol. • Protects RhoA, particularly GTP-bound form, from proteasome-mediated degradation[24] • PKC → T127 and S188 — translocation to the plasma membrane. Protects GTP-bound RhoA from proteasome-mediated degradation[128] • ERK2 — required for FBXL19-dependent RhoA degradation[47] • SLK → S188 — might inhibit RhoA activity[139]	• #SMURF1 → K6, K7 and K51 — proteasomal degradation[48,49,51,140] • §SCF^{FBXL19} → K135 — proteasomal degradation in an ERK-dependent manner[47] • $CUL3^{BACURD}$ — proteasomal degradation of GDP-bound inactive RhoA[52] • SCF^{FBXW7} — proteasomal degradation[141]	—
RhoB	CK1 → S185 — increases GDP-bound inactive form[142]	• $CUL2^{RBX1}$ — proteasomal degradation leading to liver carcinogenesis[143] • SMURF1 → K6 and K7 — degradation as part of DNA damage response pathway. RhoB abundance controls cell fate in response to DNA damage[56]	—
RhoC	AKT → S73 — required for downstream signalling and mediates increased breast cancer cell invasiveness[144]	—	—
RND1	? → S228 — regulates 14-3-3 protein binding[38]	—	—
RND2	? → S223 — regulates 14-3-3 protein binding[38]	—	—
RND3	• ROCK1 → S240 and S218 — regulates binding to 14-3-3 proteins, where RND3 is translocated to the cytosol[38] • PKC → S210 — regulates 14-3-3 binding[38]	SCF^{SKP2} → K235 — proteasome-mediated degradation to control cell cycle progression[145]	—
RhoU	SRC → Y254 — translocation from the plasma membrane to endosomes[39]	$CUL5^{RAB40A}$ → K177, K248 — protein degradation, RhoU protected by PAK4 binding to promote focal adhesion turnover[133]	—
RhoBTB2	—	CUL3 ubiquitin ligase complex — proteasome-mediated degradation[58]	—
RhoH	‡LCK? → Y73, Y83 — Tyr phosphorylation of ITAMs might modulate interaction of RhoH with ZAP70 in T cells[41]	—	—

aPKC, atypical protein kinase C; BACURD, BTB/POZ domain-containing adaptor for CUL3-mediated RhoA degradation protein 1; CDK5, cyclin-dependent kinase 5; cIAP1, cellular inhibitor of apoptosis 1; CK1, casein kinase 1; CUL3, cullin 3; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GLUT4, glucose transporter type 4; HACE1, HECT domain and ankyrin-repeat-containing E3 ubiquitin ligase 1; ITAM, immunoreceptor Tyr-based activation motif; LCK, lymphocyte-specific protein tyrosine kinase; PIAS3, protein inhibitor of activated STAT3; PKA, protein kinase A; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; RBX1, RING box 1 E3 ubiquitin protein ligase; RhoGDI, Rho GDP-dissociation inhibitor; ROCK1, Rho-associated coiled-coil-containing kinase 1; SCF^{FBXL19}, SKP1–CUL1– F box FBXL19; SLK, STE20-like Ser/Thr protein kinase; SMURF1, SMAD-specific E3 ubiquitin protein ligase 1; XIAP, X-linked inhibitor of apoptosis protein. *Data not available for RhoJ, RAC2, RhoG, RhoD, RhoF, RhoV or RhoBTB1. ‡Denotes speculative functions. §Indicates a mechanism whereby ubiquitylation is regulated by prior Rho protein phosphorylation. ‖First demonstration that a Rho GTPase undergoes sumoylation. ¶First demonstration that a Rho GTPase can undergo phosphorylation. #First demonstration that a Rho GTPase undergoes ubiquitylation.

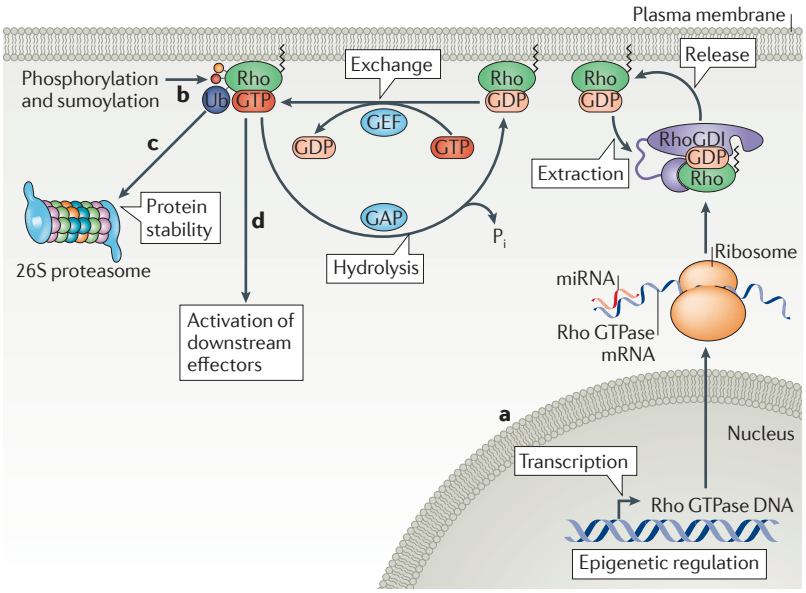


Figure 1 | **Overview of Rho GTPase regulation.** The route from Rho GTPase protein expression to effector protein activation is tightly regulated. Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) constitute the classic regulatory proteins that regulate the GTPase cycle. GEFs activate Rho GTPases by catalysing the exchange of GDP for GTP, whereas GAPs greatly stimulate the intrinsic GTPase activity of Rho GTPases and inactivate them. GDIs extract prenylated Rho GTPases from the membrane by binding the isoprenoid moiety and sequester them away in the cytoplasmic compartment. Unconventional mechanisms of regulating Rho GTPases are becoming more apparent. **a** | Rho GTPase expression can be controlled at the transcriptional level by epigenetics and at the translational level by the action of microRNAs (miRNAs). **b** | Post-translational covalent modifications of Rho GTPases, including phosphorylation and sumoylation, can result in the activation or inactivation of Rho GTPases, depending on the cellular context. **c** | Protein levels of Rho GTPases can be acutely regulated by the ubiquitin–proteasome system. **d** | The combination of classical and unconventional regulatory mechanisms ensures the appropriate spatiotemporal activation of the Rho GTPases during various cellular processes, including regulation of cytoskeletal dynamics, cell polarity and survival.

phosphorylation and sumoylation regulate GTPase activity, and ubiquitylation is important for modulating Rho GTPase protein levels.

Lipid modifications mediate Rho GTPase association with membranes. Rho GTPases are post-translationally modified by lipids (FIG. 2), which localize them to distinct membrane compartments and directly influence their interactions with specific RhoGEFs and subsequent downstream signalling pathways. The only exceptions are RhoBTB1 (Rho-related BTB domain-containing protein 1) and RhoBTB2, which interact with cullin 3 (CUL3) ubiquitin ligase complexes, seem to be tumour suppressors and are not known to be modified by lipids[7]. Polybasic residues near the carboxyl termini of some Rho GTPases contribute to membrane association[8].

The most frequent PTM of Rho GTPases is C-terminal prenylation, which involves the addition of a farnesyl (15-carbon chain) or geranylgeranyl (20-carbon chain) moiety to a Cys residue in the CAAX motif[9,10] and is mediated by farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) enzymes, respectively. Prenylation is generally followed by proteolysis of the terminal three residues and carboxymethylation of the Cys residue[8]. Although prenylation is an irreversible modification, the entry of some Rho GTPases into the different prenylation pathways can be regulated by the chaperone protein small GTPase guanosine diphosphate dissociation stimulator (SmgGDS)[11,12] (BOX 1).

In addition to prenylation, several Rho proteins can undergo *S*-palmitoylation[13–16]. Palmitoylation is a reversible process that enables GTPases to interact dynamically with membranes and potentiates downstream signalling[17,18]. For example, RAC1, which stimulates actin polymerization at the plasma membrane, is palmitoylated at Cys178 only after it has undergone prenylation at Cys189 (REF. 19). Palmitoylation increases the stability of RAC1 by targeting it to actin cytoskeleton-linked, detergent-resistant membrane regions[19]. Moreover, non-palmitoylated RAC1 is less active, and cells expressing a palmitoylation-deficient form of RAC1 show defects in cell spreading and migration[19].

Palmitoylation has also been described for the brain-specific splice variant of CDC42 (bCDC42)[20]. CDC42 has many cellular functions, including regulation of cell polarity, extension of filopodia and vesicle trafficking. The bCDC42 isoform terminates with a CCIF sequence and, after prenylation at Cys188, bypasses C-terminal proteolysis and carboxymethylation and undergoes palmitoylation at the adjacent Cys189 residue[20]. Interestingly, this dual-lipidated bCDC42 does not interact with RhoGDI and is therefore enriched at the plasma membrane, compared to a palmitoylation-deficient form[20].

The atypical GTPases RhoU and RhoV, which regulate cell adhesion and actin organization, are exclusively modified by palmitoylation at their C-terminal CFV motif, but palmitoylation alone is not sufficient to target these GTPases to the plasma membrane[15,16]. Several other basic C-terminal residues in RhoV are crucial for establishing membrane localization[16]. This is likely to be important for RhoV signalling to effector proteins such as PAK kinases (p21-activated kinases).

Phosphorylation alters Rho GTPase activity and localization. Several Rho GTPases are modified by phosphorylation, which often occurs close to lipid modifications and can therefore alter their localization (FIG. 3; TABLE 1). On the other hand, phosphorylation of residues within the GTPase domain can affect GTP–GDP cycling and/or the interaction of the Rho GTPase with its downstream effectors.

RhoA regulates actomyosin contractility and cell cycle progression, and it was the first Rho GTPase shown to be regulated by phosphorylation[21]. RhoA is phosphorylated on Ser188, close to Cys190 of its CAAX box, by the cyclic nucleotide-dependent protein kinase A (PKA) and PKG[21–24]. This phosphorylation inhibits the activity of RhoA by enhancing its interaction with RhoGDI and its concomitant extraction from the plasma membrane[22,23,25]. This inhibitory mechanism is involved in the dynamic regulation of actin protrusions at the leading edge of migrating cells[22]. Ser188 phosphorylation may also inhibit RhoA signalling by decreasing

Prenylation
The attachment of an isoprenoid group to a carboxy-terminal Cys residue or residues. An isoprenoid is a compound that is derived from 5-carbon isoprene (2-methyl-1,3-butadiene) units, which can be linked together in a head-to-tail or a tail-to-tail conformation and include farnesyl diphosphate or geranylgeranyl diphosphate molecules.

Chaperone protein
A protein that contributes to the folding or unfolding of other proteins, and/or the assembly of multiprotein complexes.

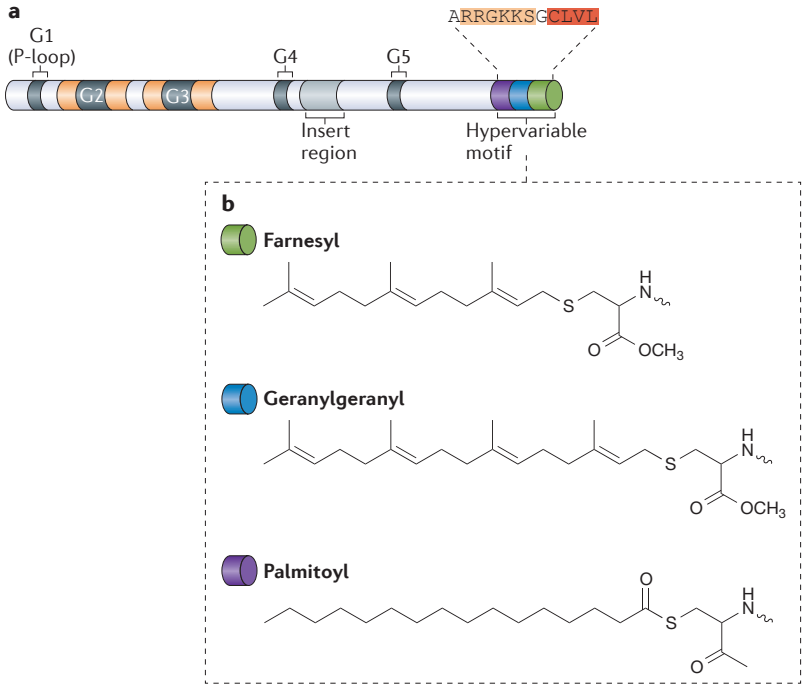


Figure 2 | **Regulation of Rho GTPases by lipid modifications. a** | A general schematic diagram of Rho GTPase domain architecture. The G domain in Rho GTPases is highly conserved and is responsible for binding to guanine nucleotides. The G domain contains a variety of amino acid motifs responsible for GTP and GDP binding and coordinating conformational changes. The P-loop (phosphate-binding loop), also known as the G1 domain, is a conserved GXXXXGKS/T motif that is responsible for binding to the β,γ-phosphate of the guanine nucleotide. Rho GTPase specificity is imparted through the hypervariable domain at the carboxyl terminus, which contains several important sequences (and shows the highest level of variability between Rho proteins). The highlighted sequence shown is taken from RhoA as an example. The CAAX motif (red) at the C terminus undergoes a variety of post-translational lipid modifications that are crucial for membrane targeting. They consist of isoprenylation, including farnesylation (green), geranylgeranylation (blue) and palmitoylation (purple). The lipid moiety tethers the Rho GTPase to the membrane and prevents free diffusion through the cytoplasm. Some Rho GTPases also contain a C-terminal polybasic region (orange) that immediately precedes the CAAX motif, containing several Lys and Arg residues, which provide a positively charged interface for membrane association. **b** | The chemical structures of the farnesyl and geranylgeranyl isoprenoids and the palmitate group, attached to Cys residues of Rho GTPases, are shown.

its interaction with the effector protein Rho-associated coiled-coil-containing kinase (ROCK)[26,27]. This mechanism was reported to be responsible for the decreased phosphorylation of myosin regulatory light chain (MRLC) by myosin light chain phosphatase (MLCP) following compressive stress on C2C12 cells[26].

Interestingly, PKG-mediated phosphorylation of RhoA was reported to induce the release of RAC1 from RhoGDI and the subsequent translocation of RAC1 to the plasma membrane, by effectively 'trapping' RhoGDI on RhoA[28]. This led to RAC-mediated stimulation of migration and adhesion of vascular smooth muscle cells (VSMCs), increasing wound repair of VSMC monolayers[28]. Furthermore, in VSMCs, phosphorylation on Ser188 protects RhoA from ubiquitin-mediated proteasomal degradation by promoting its interaction with RhoGDI[29], although presumably it is still inactive, as it is bound to RhoGDI. The phosphorylation-mediated regulation of RhoGDI interaction with RhoA is therefore a complex and coordinated process that regulates cell motility in several ways. We anticipate that this means of regulation will extend to other cell types and GTPases.

RAC1 is regulated by phosphorylation, which seems to have a profound impact on RAC1-mediated effects on the actin cytoskeleton and adhesion, thereby affecting cell morphology[30]. RAC1 phosphorylation on Tyr64 by SRC or focal adhesion kinase (FAK) affects endothelial cell spreading on fibronectin *in vitro*[30]. Expression of a non-phosphorylatable form of RAC1, RAC1-Y64F, increased the spread area of endothelial cells and increased GTP-binding. By contrast, the RAC1-Y64D phosphomimetic variant induced cell rounding with no well-developed lamellipodia or focal adhesion complexes[30]. Furthermore, phosphorylation of RAC1 by AKT on Ser71 has been shown to increase the levels of the GDP-bound form[31] (FIG. 3). Tyr64 and Ser71 both reside within the switch II region of RAC1 (residues 57–75), so phosphorylation of either residue might induce a conformational change in the GTP-binding site or result in steric hindrance, thereby reducing GTP binding. Phosphorylation of RAC1 on Thr108 by extracellular signal-related kinase (ERK) in response to epidermal growth factor (EGF) causes RAC1 to translocate to the nucleus[32], which is proposed to sequester it away from RhoGEFs and prevent its activation during cell migration. However, RAC1 can also regulate actin polymerization[33] and transcription factors such as β-catenin[34] and nuclear factor-κB[35] in the nucleus and thus has a distinct nuclear function. In addition, enforced localization of RAC1 to the nucleus alters the balance of RAC1 and RhoA in the cytoplasm and promotes RhoA-driven invasion[33].

Several atypical GTPases are regulated by phosphorylation. The most extensively studied is RND3 (also known as RhoE), which inhibits RhoA activity, in part through p190 RhoGAP, to reduce actomyosin contractility. RND3 can be phosphorylated on different sites by ROCK1 and PKC[36,37] (FIG. 3). Phosphorylation in the C-terminal domain by either of these kinases, in conjunction with a farnesyl group, targets RND3 for binding to 14-3-3 proteins[38]. By sequestering RND3 in the cytoplasm, 14-3-3 proteins render it inactive; in effect, they act on RND3 in a similar manner to RhoGDI proteins. RND1 and RND2 are also phosphorylated on equivalent Ser residues within their C termini and interact with 14-3-3 proteins, and so they are likely to be regulated by the same mechanism[38].

Similarly, the localization of RhoU (also known as WRCH1) is controlled through SRC-mediated phosphorylation of Tyr254, close to the C terminus[39], which induces a rapid translocation of RhoU from the plasma membrane to endosomes, accompanied by a reduction in the levels of active protein[39] (FIG. 3). A phosphorylation-deficient RhoU mutant is more active than wild-type RhoU in disrupting cyst formation in 3D. Phosphorylation might reduce RhoU activity by inducing its translocation away from GEFs located at the plasma membrane, although no GEF for RhoU has so far been identified. RhoH can also be Tyr phosphorylated within a non-canonical immunoreceptor Tyr-based

S-palmitoylation
Palmitoylation is the process by which a 16-carbon palmitate group is added to a Cys residue by a palmitoyltransferase enzyme. In most cases, palmitate is attached through reversible thioester linkage (*S*-palmitoylation).

Actomyosin contractility
A process whereby myosin II filaments interact with and move along anti-parallel actin filaments.

CAAX box
A conserved carboxy-terminal amino acid recognition motif, in which C is Cys, A is an aliphatic amino acid and X is variable. The motif is recognized by prenyltransferases that attach an isoprenoid moiety to the Cys residue.

14-3-3 proteins
14-3-3 proteins are homodimeric proteins that bind to a wide range of intracellular proteins, usually by interacting with phosphorylated Ser or Thr residues.

26S proteasome
A protein complex found in mammalian cells that degrades proteins by proteolysis. Polyubiquitylation is a common signal for proteasome-mediated degradation.

activation motif (ITAM), which induces its interaction with the Tyr kinase ZAP70 in T cells and thereby increases T cell receptor signalling[40,41].

Phosphorylation of Rho GTPases provides another layer of regulation in addition to GTP–GDP cycling and, in the case of atypical GTPases such as RND proteins, provides the major mechanism for regulating their activity. Atypical GTPases contain unique amino-terminal and C-terminal extensions at either end of the core GTP-binding domain, which probably impart a greater level of specificity by providing more phosphorylation sites[42].

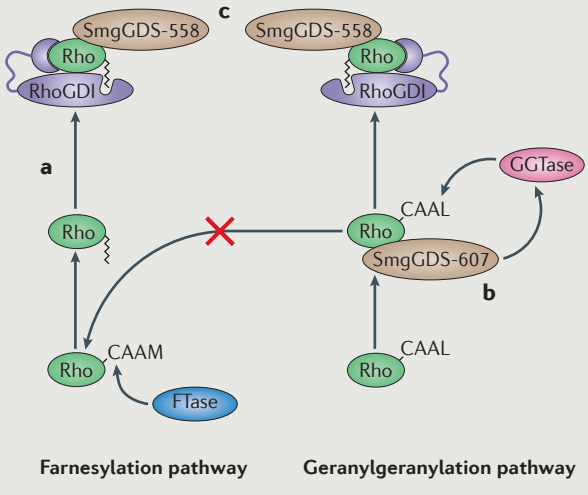
Ubiquitylation and sumoylation regulate Rho GTPase turnover and activity. Most Rho GTPases are probably regulated by ubiquitylation (see the PhosphoSitePlus resource)[43], although only the ubiquitylation of RhoA and RAC1 have been characterized in detail. Ubiquitylation is a three-step process that involves the covalent addition of ubiquitin polypeptides to cellular proteins through the activity of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes[44,45] (BOX 2). A chain of four or more ubiquitin monomers — polyubiquitylation — covalently linked together by their Lys48 residues marks these proteins for degradation by the 26S proteasome[46], thereby regulating protein turnover. Monoubiquitylation or polyubiquitylation through other ubiquitin Lys residues have other effects on protein function, including targeting them to different subcellular compartments[44,46]. Advances in our understanding of the E3 ligases that catalyse the ubiquitylation of Rho GTPases have shed new light on the important function of this modification in coordinating Rho GTPase activity in precise spatial and temporal contexts.

RhoA, which is the best-characterized ubiquitylation target in the Rho family[45], is ubiquitylated by three different E3 ligase complexes: the SKP1–CUL1–F-box (SCF)^{FBXL19} complex[47], the SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) complex[48–51] and the BTB/POZ domain-containing adaptor for CUL3-mediated RhoA degradation (BACURD)–CUL3–RING ubiquitin ligase complex[52,53]. Ubiquitylation by different complexes could be an important mechanism by which cells can modulate the levels of RhoA at particular intracellular sites or in response to specific signals. For example, PKCζ-mediated recruitment of SMURF1 to the leading edge of migrating cells leads to the ubiquitylation of RhoA on Lys6 and Lys7, followed by its localized proteasome-mediated degradation[48,49] (reviewed in REF. 45). This degradation prevents RhoA from stimulating the formation of stress fibres at the leading edge and thereby facilitates the RAC-driven protrusion of lamellipodia. Increased SMURF1 expression reduces the levels of RhoA and stimulates cancer cell migration, invasion and metastasis[54], but whether the reduction in RhoA is localized or global is unclear. During axon development, PKA-mediated phosphorylation of SMURF1 increases its ability to degrade RhoA. This removes the growth-inhibitory effects of RhoA, resulting in axon extension[55]. SMURF1 also targets RhoB for degradation, and a reduction in SMURF1 levels in response to DNA damage therefore promotes RhoB-mediated apoptosis[56].

SCF^{FBXL19}-mediated RhoA degradation similarly decreases the phosphorylation of myosin light chain (MLC) in lung epithelial cells[47], which is expected to reduce actomyosin contractility. Intriguingly, it was also

Box 1 | SmgGDS controls the entry of Rho GTPases into the prenylation pathway

Although prenylation is an irreversible modification, the entry of Rho GTPases into the different prenylation pathways is regulated by the scaffolding protein small GTPase guanosine diphosphate dissociation stimulator (SmgGDS)[11,12]. SmgGDS exists as two different splice variants: SmgGDS-558 and SmgGDS-607 (REF. 12). When Met constitutes the carboxy-terminal residue in the CAAX box, the Rho GTPase enters the farnesylation pathway, before being trafficked by the SmgGDS-558 variant to the plasma membrane (see the figure, part **a**).
The SmgGDS-607 splice variant is unable to recognize Met, but it does recognize and interact with the terminal Leu residue of non-prenylated GTPases that will go through the geranylgeranylation pathway, and presents the GTPase to a geranylgeranyltransferase (GGTase) enzyme for modification (see the figure, part **b**). Hence, this model implies that the enzyme activity of the farnesyltransferase (FTase) is higher than that of the geranylgeranyltransferase, and the role of the SmgGDS-607 variant is to prevent inappropriate farnesylation of Rho GTPases by sequestering them away until a geranylgeranyltransferase can modify them[12,134].
Once prenylated, the Rho GTPase is bound by the SmgGDS-558 variant and delivered to the plasma membrane through the action of RhoGDIs, which protects the Rho GTPase from degradation and protein misfolding (see the figure, part **c**).
Interestingly, phosphorylation of RAP1B on a Ser residue adjacent to its CAAX box decreases its interaction with SmgGDS, with a concomitant reduction in its prenylation[135], raising the possibility that C-terminal modifications on Rho GTPases could also regulate their interaction with SmgGDS proteins.



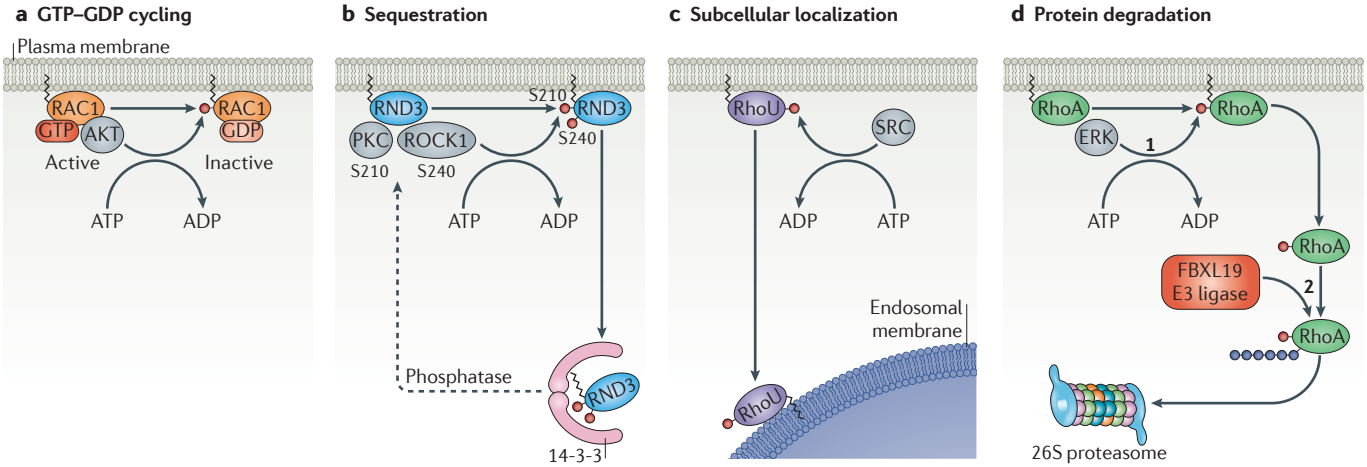


Figure 3 | **Phosphorylation has diverse effects on Rho GTPase signalling.** Phosphorylation of Rho GTPases can signal for various regulatory mechanisms. **a** | GTP—GDP cycling. The phosphate group can affect the ability of Rho proteins to bind to guanine nucleotides. AKT-mediated phosphorylation of RAC1 results in a decrease in GTP binding. **b** | Sequestration. Phosphorylation can signal for the extraction of Rho GTPases from the plasma membrane by RhoGDIs or 14-3-3 proteins. The phosphate group functions as a recognition module for the extractor protein, or it can induce a conformational change in the Rho GTPases that increases binding. For example, the phosphate group is used in tandem with the prenyl moiety to form a dual recognition module at the carboxy-terminal domain of RND3 for 14-3-3 protein binding. **c** | Subcellular localization. Phosphorylation can induce the translocation of the Rho protein to different cellular compartments. However, the specific mechanism by which RhoU is translocated in the endocytic compartment by SRC-mediated phosphorylation remains elusive. **d** | Protein degradation. Phosphorylation can provide an additional level of specificity in targeting Rho proteins for degradation, as has been shown for RhoA. Extracellular signal-related kinase (ERK) first phosphorylates RhoA at an unknown residue (step 1), which presumably induces a conformational change that reveals the Lys135 acceptor site for ubiquitylation (step 2). PKC, protein kinase C; ROCK1, Rho-associated coiled-coil-containing kinase.

demonstrated that ubiquitylation depended on the prior ERK2-mediated phosphorylation of RhoA[47] (FIG. 3). This interplay between different PTMs imparts a greater level of specificity to Rho GTPase regulation.

BACURDs specifically bind to RhoA and link it to RING E3 ligases via the scaffold CUL3. Depletion of CUL3 or BACURD stimulates the assembly of actin stress fibres in HeLa cells by increasing RhoA levels[52]. BACURDs might control the spatial and temporal activation of RhoA[52]. RhoBTB2 and RhoBTB3 bind directly to CUL3 through a BTB domain-mediated interaction[57,58] and are themselves ubiquitylated by CUL3-dependent ubiquitin ligase complexes, although the significance of this is not yet clear[57,58].

RAC1 is also subject to ubiquitylation and degradation by a range of E3 ligases, including inhibitor of apoptosis (IAP) proteins (X-linked IAP (XIAP) and cellular IAP1 (cIAP1))[59,60], HACE1 (REFS 61–64) and SCF^{FBXL19} complexes[65]. Interestingly, RAC1 is polyubiquitylated on Lys147 by IAPs and HACE1, whereas SCF^{FBXL19} polyubiquitylates RAC1 and its close relative RAC3 on Lys166 (REFS 65,66), suggesting that different E3 ligases ubiquitylate RAC1 depending on the subcellular localization and/or stimulus, as for RhoA. Ubiquitylation by these three E3 ligases targets RAC1 for degradation, which affects cell morphology and migration[59,62,65]. For example, downregulation of cIAP1 and XIAP resulted in an elongated morphology and enhanced cell migration in both normal and tumour cells, owing to increased RAC1 signalling[59].

RAC1 is the only Rho GTPase to date that has been reported to undergo sumoylation[67]. Sumoylation does not seem to be essential for RAC1 activation, but it helps to maintain RAC1 in an activated state. The E3 SUMO ligase PIAS3 (protein inhibitor of STAT3) sumoylates RAC1 on several C-terminal Lys residues in response to stimulation by hepatocyte growth factor (HGF), which increases its GTP binding ability and enhances the formation of lamellipodia and cell migration[67]. RAC1 therefore seems to be modified in various ways, all of which influence its ability to regulate cellular processes. The cumulative effect of these modifications might shift the activity of RAC1 above or below a certain threshold, allowing RAC1 responses to be finely tuned spatiotemporally. We anticipate that other Rho proteins are regulated by similar combinatorial PTMs.

Regulation of GEFs

GEFs are classified into two families, DBL and DOCK (dedicator of cytokinesis), according to their structure[4,68]. DBL-family GEFs, which constitute the majority of Rho GEFs, contain a DBL-homology (DH) domain associated with a pleckstrin homology (PH) domain — the so-called DH–PH domain — flanked by regions that confer specificity towards particular Rho GTPases[68]. DOCK family GEFs possess two conserved domains: DOCK homology region 1 (DHR1), which, in some cases, mediates membrane localization owing to the presence of a lipid-binding C2 domain; and DHR2, which is the catalytic GEF domain[69].

BTB domain
A domain that was identified in 'Broad-complex, Tramtrack and Bric-a-brac' proteins in *Drosophila melanogaster* and that is usually located in the amino-terminal region of proteins. Originally known as the POZ domain, this motif is found in several virus proteins. Many BTB-domain proteins contain a second protein–protein interaction motif, such as zinc-finger or Kelch motifs.

DBL-homology (DH) domain
A domain of approximately 200 amino acids that induces release of GDP from Rho GTPase family members.

Pleckstrin homology (PH) domain
A domain of approximately 120 amino acids. Some pleckstrin homology domains bind to headgroups of membrane lipids on cellular membranes and recruit intracellular signalling proteins to specific compartments.

Post-translational modifications alter RhoGEF activity. RhoGEFs are often regulated by phosphorylation, which usually results in activation of the GEF, GDP–GTP exchange and Rho GTPase activation; however, in some cases, phosphorylation inhibits GEF activity. Phosphorylation alters the activity of GEFs, either by inducing conformational changes in the catalytic domain or by regulating GEF binding to scaffolding proteins (TABLE 2). The best-characterized examples are the three VAV proteins, which are activated as GEFs by Tyr phosphorylation[70]. Similarly, several studies have revealed that ERK-mediated phosphorylation of GEF-H1 (also known as ARHGEF2) at Thr678 stimulates its nucleotide exchange activity towards RhoA[71–73]. Interestingly, integrin stimulation by mechanical force has been shown to increase ERK-mediated phosphorylation of GEF-H1 and its subsequent recruitment to focal adhesion complexes[73]. Phosphorylation of RhoGEFs also creates docking sites for 14-3-3 proteins[74–77]. In most cases, 14-3-3 proteins inhibit GEF activity — in the case of GEF-H1, by sequestering it on microtubules[74] — but they have also been shown to stimulate RhoGEF function by altering subcellular localization. For example, 14-3-3ζ binds to and recruits T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1) to β1 integrin-containing adhesion complexes, where it can activate RAC1 and facilitate cell motility[77]. TIAM1 is phosphorylated by various kinases, which regulate its activity, stability and localization[78–80] (TABLE 2). For instance, during mitosis, TIAM1 is phosphorylated by cyclin-dependent kinase 1 (CDK1) at Ser1466 on centrosomes. During prophase, this results in activation of RAC1 and, subsequently, of the group I PAKs, PAK1 and PAK2 (REF. 79). PAK1 and PAK2 antagonize each other during centrosome separation to ensure a correct mitotic exit[79].

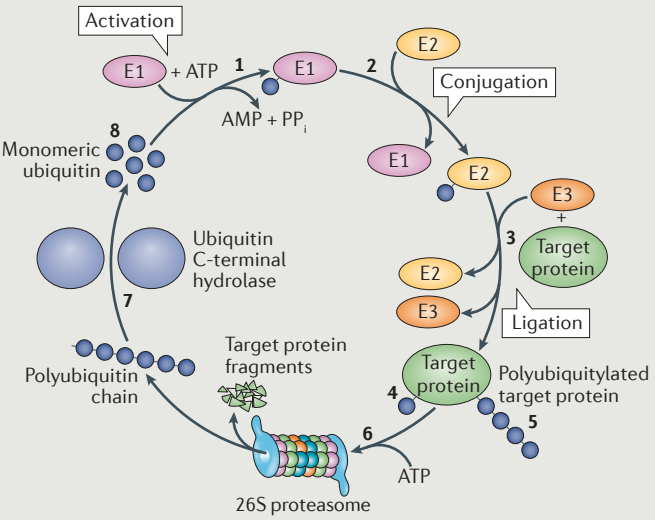
Box 2 | Ubiquitin and sumoylation cycles

Ubiquitylation and sumoylation use analogous enzymatic cascades involving an E1 activating, E2 conjugating and E3 ligase enzyme to modify their targets.

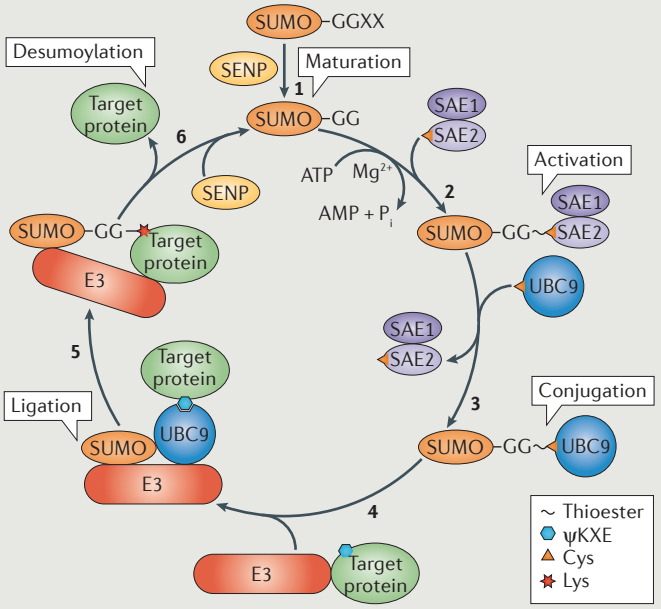
In the case of ubiquitylation, E1 activating enzymes first activate the 76-amino-acid ubiquitin by adenylation in an ATP-dependent reaction, before it is conjugated to a Cys residue on the enzyme (step 1 in the figure, part **a**)[44]. Ubiquitin is then transferred from the E1 enzyme to the E2 conjugating enzyme (step 2)[44]. E3 ubiquitin ligases, which provide substrate specificity, covalently attach ubiquitin to Lys residues in the target protein (step 3)[44,45]. E3 ligase enzymes fall into four main classes: HECT-type, RING-finger type, U-box type and PHD-finger type[45]. Proteins can be monoubiquitylated (single ubiquitin molecule; step 4) or polyubiquitylated (multiple ubiquitin molecules in a chain; step 5). Polyubiquitylation occurs predominantly on Lys48 residues within ubiquitin chains and is associated with degradation by the 26S proteasome (step 6)[44]. Other polyubiquitylation (such as on K69) and monoubiquitylation events are associated with cellular processes such as DNA repair or mediate subcellular localization. After proteasome-mediated degradation, ubiquitin chains are cleaved by ubiquitin carboxy-terminal hydrolase (step 7). Monomeric ubiquitin can then re-enter the cycle (step 8).

Small ubiquitin-related modifiers SUMO1, SUMO2, SUMO3 and SUMO4 first undergo processing by sentrin-specific proteases (SENPs; step 1 in the figure, part **b**). The mature form of SUMO possesses a C-terminal GG motif that becomes conjugated to a Cys residue in a SUMO-activating enzyme 1 (SAE1)–SAE2 complex[136]. Before conjugation, the SAE1–SAE2 complex catalyses the adenylation of SUMO (step 2). In step 3, SUMO is transferred from SAE1–SAE2 to the Cys residue of the E2 conjugating enzyme, ubiquitin-like conjugating enzyme 9 (UBC9)[136]. E3 SUMO ligases then transfer the SUMO group from UBC9 to a Lys residue in the target protein, often in the SUMO consensus motif ΨKXE (where Ψ represents any hydrophobic amino acid and X denotes any amino acid; steps 4 and 5). E3 SUMO ligases include PIAS (protein inhibitor of STAT) proteins and SIZ proteins. Sumoylation does not signal for protein degradation but instead affects processes such as transcriptional regulation and nuclear transport[136]. SUMO groups are deconjugated from target proteins by SENPs, and free SUMO can be reconjugated (step 6).

a The ubiquitylation pathway



b The sumoylation pathway



RhoGEFs are subject to precise temporal regulation during distinct stages of cellular processes to ensure that cell morphologies are controlled at the appropriate steps. For example, cytokinesis is associated with the formation of an actomyosin cleavage furrow, which is dependent on RhoA activity, but actomyosin contractility would be detrimental to mitotic cell rounding in early mitosis[81]. Accordingly, GEF-H1 is phosphorylated in early mitosis by both the Aurora A and CDK1–cyclin B kinases at Ser885, which inhibits its activity. However, in telophase, GEF-H1 exists in a dephosphorylated, active form, which promotes RhoA activation at the cleavage furrow and the assembly of the actomyosin contractile ring[81]. Leukaemia-associated RhoGEF (LARG) is regulated in a similar manner by CDK1-dependent phosphorylation to ensure that RhoA activity is high during cytokinesis[82]. Finally, the localization of MyoGEF at the cleavage furrow is also regulated by Aurora B kinase[83]. Hence, several distinct mechanisms localize a range of RhoGEFs to the cleavage furrow to ensure a transient localized spike of RhoA activity during cytokinesis.

Temporal regulation of RhoGEFs also occurs during the development and growth of sensory neuronal axons[84]. During early embryonic development in mice, while axons are extending, protein phosphatase 2A (PP2A) dephosphorylates and activates DOCK6, which promotes axon growth by increasing RAC1 activity. Later, stimulation of TRKA (also known as high-affinity nerve growth factor receptor) leads to the activation of AKT, which phosphorylates DOCK6, thereby inhibiting its GEF activity and axon extension[84]. The growth of axons is therefore terminated once they have reached their targets by the inhibition of DOCK6 at the correct developmental stage.

Like Rho GTPases, several RhoGEFs can be targeted for degradation by ubiquitylation (TABLE 2). For example, in response to HGF stimulation, E3 ubiquitin protein ligase HUWE1 (HECT, UBA and WWE domain containing 1) catalyses the ubiquitylation and degradation of TIAM1 at sites of cell–cell adhesion[85]. The decrease in active TIAM1 results in the disassembly of the junctions and promotes an invasive phenotype in MDCKII epithelial cells[85]. The RhoA GEF ephexin 5 is ubiquitylated in response to ephrin type-B receptor 2 (EphB) stimulation in neuronal cells[86]. Under basal conditions, ephexin 5 inhibits excitatory synapse development by restricting the growth and number of dendritic spines. However, upon ephrin B activation, the EphB receptor triggers the phosphorylation of ephexin 5 (REF. 86). Phosphorylated ephexin 5 is a substrate for ubiquitin protein ligase 3A (UBE3A)-mediated ubiquitylation and degradation, which promotes excitatory synapse development[86]. This maintains the correct balance of excitatory synapse numbers in the developing brain.

Finally, a RhoGEF was recently shown to undergo acetylation. Two nuclear localization sequences (NLSs) near the N terminus of NET1A (neuroepithelial cell-transforming gene 1 isoform A; also known as ARHGEF8) promote its sequestration in the nucleus; however, acetylation of residues around the second NLS in response to EGF direct its translocation to the cytoplasm, where it is then able to activate RhoA[87].

Coordination of Rho GTPase signalling through GEF-containing complexes. In response to extracellular stimuli, many GEFs interact with specific proteins, including targets of Rho GTPases, which help to coordinate Rho GTPase signalling at specific sites in cells. A well-known example is a complex containing the RAC/CDC42-specific GEF β-PIX with the RAC/CDC42 target PAK, which regulates turnover of integrin-containing focal adhesions (see REF. 88 for a review). In addition, in response to HGF signalling, the RAC/CDC42-specific GEF ASEF (APC-stimulated GEF; also known as ARHGEF4) forms a functional complex with the RAC/CDC42 target IQGAP1 (REF. 89). IQGAP1 is able to bind to ARP3 (actin-related protein 3) and cortactin, thereby linking RAC1 effectors responsible for cortical actin polymerization with ASEF and active RAC1. This leads to the remodelling of peripheral actin filaments in endothelial cells[89]. Another example is the RAC GEF P-REX1, which brings RAC1 together with its target FLI1 (friend leukaemia integration 1 transcription factor) to stimulate cell migration[90]. Similarly, DOCK1 is recruited to sites of developing cell–cell contacts by ELMO2 (engulfment and cell motility protein 2)[91]. Both proteins are necessary to induce the recruitment of E-cadherin and to coordinate localized RAC1 activation and the actin cytoskeleton rearrangements that are required to strengthen these intercellular contacts. After this process has finished, DOCK1 dissociates from the ELMO2 scaffold[91].

The assembly of protein complexes can also protect GEFs from degradation. For example, the interaction of NET1 with Discs-large homologue 1 (DLG1) increases NET1 stability[92,93]. Interestingly, this interaction is regulated by cell–cell contact, as an increase in the ubiquitylation of NET1 occurred when cell–cell contacts were disrupted[92]. Similarly, dynamin 2 forms a complex with the GEF VAV1, which promotes VAV1 stability; disrupting this interaction targets VAV1 for lysosomal degradation through an interaction with HSC70 (REF. 94).

Some GEFs are regulated by forming homo-oligomers and hetero-oligomers[95,96]. PDZ-RhoGEF (also known as ARHGEF11), LARG and p115 RhoGEF belong to a RhoGEF subfamily that is characterized by the presence of a C-terminal RGS-like (RGL) domain. PDZ-RhoGEF and LARG can homo- and hetero-oligomerize, whereas p115-RhoGEF only forms homo-oligomers. Oligomerization occurs through the RGL domain, which, if removed, increases nucleotide exchange activity and results in RhoA activation[95]. Hence, oligomerization might be a mechanism to self-inhibit RhoGEF activity. Similarly, oligomerization of two C-terminal Leu zipper motifs decreases the GEF activity of AKAP-LBC (also known as AKAP13)[96]. Oligomerization is necessary for AKAP-LBC to bind to 14-3-3 proteins (outlined above)[96], where it presumably forms an inhibitory tetrameric complex.

Regulation of GAPs

Members of the RhoGAP family contain a conserved 150-residue RhoGAP domain, which mediates

Table 2 | **Post-translational modifications of GEFs, GAPs and GDIs**

	Phosphorylation	Ubiquitylation	Sumoylation
RhoGEFs			
VAV	See recent review[70]	CBL — targets VAV2 for degradation, resulting in reorganization of junctional actin cytoskeleton[146]	—
TIAM1	• CK1 → S239, S334, T340 — triggers SCF-mediated ubiquitylation and reduces mTOR-S6K signalling pathway[78] • CDK1 → S1466 — activates PAK kinases on centrosomes during prophase[79] • SRC → Y384 — adherens junction disassembly[80] • AKT → S60, S172, S231, S695 — induces 14-3-3 protein interaction and promotes TIAM1 stability[76]	• $CUL3^{KBTBD6/7}$ → K1404/K1420 — targets TIAM1 for proteasome-mediated degradation, dependent on binding to GABARAP proteins[147] • $SCF^{\beta TrCP}$ — targets TIAM1 for proteasome-mediated degradation and may control mTOR-S6K signalling[78] • HUWE1 → K595 — targets TIAM1 for proteasome-mediated degradation predominantly at cell–cell adhesions in response to HGF stimulation[85]	—
LARG	• FAK — links G protein-coupled receptors to Rho activation[148] • CDK1 → S190, S1176 — inactivates LARG as cells enter mitosis[82]	? — deubiquitylation by CYLD[149]	—
GEF-H1	• Aurora A/B → S885, CDK1/cyclin B → S959 — inhibits GEF activity, resulting in a decrease in RhoA-induced contractility at the cleavage furrow during mitosis[81] • PAK1 → S885 — induces 14-3-3 protein interaction and relocation to microtubules[74] • ERK1/2 → T678 — enhances GEF activity towards RhoA[71] • PAR1B → S885, S959 — inhibits GEF activity towards RhoA, preventing stress fibre formation[150]	—	—
DBL	Growth factor stimulation → Y510 — enhances GEF activity towards CDC42, RAC1 and RhoA[151]	CHIP — targets DBL for proteasome-mediated degradation[152]	—
ECT2	PKCι → T328 — enhances GEF activity towards RAC1 and drives cell growth and invasion[153]	APC^{CDH1} — targets ECT2 for proteasome-mediated degradation after mitosis[154]	—
PDZ-RhoGEF	• PAK4 — inhibits GEF activity towards RhoA[155] • FAK — links G protein-coupled receptors to Rho activation[148]	$CUL3^{KLHL20}$ — targets PDZ-RhoGEF for proteasome-mediated degradation and controls neurotrophin-induced neurite outgrowth[156]	—
Ephexin5	EphB2 → Y361 — triggers UBE3A-mediated degradation[86]	UBE3A — targets ephexin 5 for proteasome-mediated degradation and promotes EphB-dependent excitatory synapse development[86]	—
RhoGAPs			
DLC1	• PKD → S807 — does not affect *in vitro* GAP activity but negatively regulates cellular function[157] • CDK5 → S120, S205, S422, S509 — relieves auto-inhibition and activates DLC1 (REF. 101) • ERK1/2 → T301, S308 — enhances GAP activity by providing binding sites for PP2A interaction and subsequent dephosphorylation[158]	$CRL4A^{FBXW5}$ — targets DLC1 for proteasome-mediated degradation which promotes non-small cell lung cancer anchorage independent growth[99]	—
CdGAP	• GSK3 → T776 (REF. 159) • ERK1/2 → T776 — inhibits GAP activity[160]	—	—
MgcRACGAP	Aurora B → S387 — phosphorylation switches MgcRACGAP from a RAC/CDC42 GAP to a RhoGAP at the mid-body during cytokinesis[161]	APC^{CDH1} — targets MgcRACGAP for proteasome-mediated degradation during late M phase of the cell cycle[162]	—
p190A RhoGAP	• PDGF receptor → Y308 — releases TFII from p190A, allowing it to translocate to the nucleus and activate transcription[163] • ROCK → S1150 — enhances GAP activity towards RhoA and impairs RND binding[164] • GSK3β → S1476, T1480 — inhibits GAP activity and contributes to cellular polarization during directional cell migration[165] • ERK → S1476, T1480 — inhibits GAP activity resulting in Rho-dependent focal adhesion formation[166]	APC^{CDH1} — targets p190A for proteasome-mediated degradation and regulates cellular motility[167]	—

Table 2 (cont.) | **Post-translational modifications of GEFs, GAPs and GDIs**

	Phosphorylation	Ubiquitylation	Sumoylation
RhoGDIs			
RhoGDI1	• SRC → Y27, Y156 — reduces RhoA, RAC1 and CDC42 complex association[121] • PKCα → S34 (REF. 122), S96 (REF. 123) — releases RhoA from RhoGDI • PAK1 → S101, S174 — dissociation of RhoGDI from RAC1 but not RhoA[124] • PKA → S174 — probably induces a stable RhoA–RhoGDI complex[126] • FER — prevents RAC1 binding, but does not induce release of RAC1 from established complexes[125]	GRAIL — specific inhibition of RhoA activation[168]	? → K138 — increases Rho complex association[127,128]
RhoGDI2	• SRC → Y153 — reduces complex association with RAC1 (REF. 169) • PKCα → S31 — reduces complex association with RAC1 (REF. 170)	GRAIL — specific inhibition of RhoA activation[168]	—

Owing to space limitations, only regulatory proteins that are cited in the literature more than once are included in this table. APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; CHIP, carboxyl terminus of Hsc70-interacting protein; CK1, casein kinase 1; $CRL4A^{FBXW5}$, cullin 4A RING ubiquitin ligase–F-box and WD repeat domain containing 5; CYLD, cylindromatosis; DLC1, deleted in liver cancer 1; EphB2, ephrin type-B receptor 2; ERK, extracellular signal-related kinase; FAK, focal adhesion kinase; GABARAP, γ-aminobutyric acid receptor-associated protein; GAPs, GTPase-activating proteins; GDIs, guanine nucleotide-dissociation inhibitors; GEFs, guanine nucleotide exchange factors; GRAIL, gene related to anergy in lymphocytes; GSK3, glycogen synthase kinase 3; HGF, hepatocyte growth factor; HUWE1, HECT, UBA and WWE domain containing 1; KBTBD6/7, Kelch repeat and BTB (POZ) domain containing 6/7; KLHL20, Kelch-like protein 20; LARG, leukaemia-associated RhoGEF; PAK, p21-activated kinase; PAR1B, polarity-regulating kinase partitioning-defective 1B; PDGF, platelet-derived growth factor; PKC, protein kinase C; PKD, protein kinase D; SCF, SKP1–CUL1– F box; TF, transcription factor; TIAM1, T-lymphoma invasion and metastasis-inducing protein 1; UBE3A, ubiquitin protein ligase E3A.

binding to GTP-loaded Rho proteins and stimulates their intrinsic GTP hydrolysis activity[97]. Approximately 80 members of the RhoGAP family exist in humans[97,98], which far outnumbers the 20 Rho GTPases, suggesting that several RhoGAPs can impart specialized functions to individual Rho GTPase proteins. It follows, then, that the activity of each RhoGAP must be tightly controlled to ensure an appropriate equilibrium between the GDP-bound and GTP-bound states of Rho GTPases, which is achieved by various regulatory processes.

Post-translational modifications alter RhoGAP activity. The GAP activity of several RhoGAPs is regulated by phosphorylation (TABLE 2). A number of phosphorylation sites have been characterized on the RhoGAP DLC1 (deleted in liver cancer 1), the product of a tumour suppressor gene that is lost in several cancers through either epigenetic silencing or gene deletion[99]. PKA-mediated phosphorylation of DLC1 on Ser549 induces its homodimerization, which increases its RhoGAP activity[100]. DLC1 can also be phosphorylated by CDK5 on four Ser residues — Ser120, Ser205, Ser422 and Ser509 — located N-terminal to the GAP domain, which relieves an auto-inhibitory conformation[101], presumably to enable DLC1 to homodimerize and become fully active. It is likely that other GAP proteins are also regulated by sequences outside the GAP domains that mediate protein–protein interactions and induce conformational changes in the protein structure. As another example, phosphorylation of the RAC-specific GAP FilGAP (filamin A-associated RhoGAP; also known as ARHGAP24) on Ser402, which is C-terminal to the RhoGAP domain, switches its subcellular localization from the cytoskeletal network to the cytoplasm, where it undergoes activation and subsequently inactivates RAC1 (REF. 102). This results in the decreased spreading of HeLa cells on fibronectin, and cell adhesion to fibronectin induces dephosphorylation of FilGAP at Ser402 (REF. 102).

Several RhoGAPs are regulated by ubiquitylation (TABLE 2). Ubiquitylation of DLC1 by a $CUL4^{FBXW5}$ ubiquitin ligase complex leads to its degradation in non-small-cell lung cancer cell lines[99]. This provides another mechanism, in addition to gene deletion or epigenetic silencing, to remove this tumour suppressor. On the other hand, p250GAP (also known as ARHGAP32), a GAP for RhoA, undergoes non-proteolytic ubiquitylation by the APC^{CDH1} ubiquitin ligase complex before cooperating with the SMURF1 ubiquitin ligase to reduce RhoA activity and promote axon growth in the mammalian brain[103]. Additionally, ARHGAP21 has been identified as a target for sumoylation, although whether this modification affects its GAP activity is unknown[104]. It appears that we are only just beginning to appreciate the diversity of the modifications that regulate GAPs.

GAP-containing protein complexes contribute to Rho GTPase signalling. As large, multidomain proteins, RhoGAPs contain several domains that mediate interactions with scaffolding proteins and/or cytoskeletal regulators to ensure appropriate localization and specificity towards their Rho targets. For example, SRGAP3 is targeted, through its F-BAR domain, to the plasma membrane, where it is postulated to inhibit lamellipodium formation at the leading edge through GAP-dependent inhibition of RAC1 and GAP-independent effects on lamellipodin function[105]. SRGAP3 and RAC1 interact with both lamellipodin and the WAVE regulatory complex[106], indicating that a complex protein network coordinates the regulation and function of RAC1 at the leading edge of migrating cells.

WAVE regulatory complex
A 400 kDa protein complex composed of five subunits — WAVE, HSPC300, ABI, NAP1 and PIR121 — that activates the ARP2/3 complex via the VCA domain of WAVE. This initiates the polymerization of branched actin filaments.

p190 RhoGAP, a GAP for RhoA, is recruited by p120 catenin to endothelial cell–cell contacts, where local inhibition of RhoA and reciprocal activation of RAC1 sustain endothelial cell barrier regulation[107]. Similarly, DLC1 localizes to focal adhesions through its interaction with tensin proteins, which link the actin cytoskeleton to integrins[108–110]. Tensins sequester DLC1 away from RhoA, thereby increasing RhoA activity. It might seem paradoxical to bring a GAP (DLC1) in close proximity to a GTPase that needs to remain active (RhoA), but this strategy could ensure a tight RhoA signalling zone by minimizing GAP diffusion.

DLC1 is also subject to spatiotemporal activation during cell migration through a phosphorylation switch[111]. Phosphorylation of tensin 3 (TNS3; a known DLC1-binding protein) and PTEN in response to EGF stimulation triggers the dynamic re-arrangement of TNS3–DLC1 and PTEN–PI3K complexes into TNS3–PI3K and DLC1–PTEN complexes[111]. Consequently, TNS3–PI3K localizes to the leading edge of migrating cells to promote RAC1 signalling and lamellipodium formation, presumably through PI3K–phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3)-mediated recruitment of RAC GEFs[112]. On the other hand, PTEN–DLC1 localizes to the rear of the cell. PTEN binding cannot relieve the auto-inhibited conformation of DLC1, ensuring that it does not inhibit RhoA; PTEN dephosphorylates PtdIns(3,4,5)P_3 and should thereby inhibit RAC1 signalling. This strategy ensures coordinated cytoskeletal remodelling and drives cell migration[111].

Finally, the polyubiquitin-binding domain of BARGIN, a brain-specific RhoGAP splice variant, facilitates the distribution of BARGIN to membrane compartments, where it can inactivate RAC1 (REF. 113). Intriguingly, BARGIN–polyubiquitin–RAC1 signalling might be involved in the pathogenesis of Alzheimer disease[113].

GEFs and GAPs form regulatory complexes. GEFs and GAPs can coexist in regulatory complexes to fine-tune and maintain optimal levels of Rho GTPase signalling. For instance, a balance between the RAC GEF TIAM1 and the RAC GAP breakpoint cluster region (BCR) is required to regulate RAC1 activity precisely, ensuring the correct development of excitatory synapses on dendritic spines[114].

Complexes can also form between GEFs and GAPs that act on distinct GTPases to antagonize their activity. In a 3D cell migration model, dephosphorylation of the RAC/CDC42 GEF β-PIX by PP2A is required for β-PIX to associate with SRGAP1. Consequently, SRGAP1 represses RhoA activity, whereas CDC42 activity is enhanced at the leading edge of a migrating cell to ensure efficient, coordinated migration[115].

GEFs and GAPs in a complex do not necessarily antagonize each other's functions[116]. For example, vascular endothelial growth factor (VEGF) promotes an interaction between the RAC-specific GEF DOCK4 and the CDC42 GEF DOCK9 during angiogenesis. This allows for the formation of lateral filopodial protrusions through CDC42 signalling during tubule development, and the subsequent formation of lateral cell–cell contacts between the endothelial cells, leading to proper lumen morphogenesis.

The BCR and ABR (active BCR-related) proteins can bypass the need to form complexes, as they contain both GEF and GAP domains[117]. In a single-cell wound repair model, ABR localizes to the cellular region where Rho is active; it activates Rho using its GEF domain and inactivates CDC42 through its GAP domain, thereby enabling the tight segregation of Rho and CDC42 signalling zones to facilitate rapid cell repair[117].

Regulation of GDIs

The RhoGDI protein family comprises three members — RhoGDI1, RhoGDI2 and RhoGDI3 — in mammals, with distinct patterns of expression and Rho GTPase binding[118]. RhoGDI–GTPase interactions occur in a two-step process: first, the N-terminal domain of the GDI binds to the switch domains of the Rho GTPase, which inhibits the transition between the GDP- and GTP-bound forms; subsequently, the GTPase prenyl moiety moves from the membrane to the hydrophobic pocket of the GDI C-terminal domain, mediating membrane extraction of the GTPase[119,120]. RhoGDI–GTPase binding is regulated by several mechanisms.

Dynamic regulation of RhoGDIs by post-translational modifications. Generally, phosphorylation of a RhoGDI decreases its affinity for a Rho GTPase, thereby promoting GTPase dissociation and subsequent activation by a GEF[118] (TABLE 2). For example, phosphorylation mediated by SRC, PKCα, PAK1 or FER negatively regulates RhoGDIs by decreasing their affinity for a specific Rho protein[121–125].

However, phosphorylation does not always inhibit RhoGDI function. PKA-mediated phosphorylation on Ser174 of RhoGDI1 stabilizes a RhoA–RhoGDI1 complex, thereby inhibiting RhoA signalling[126]. This mechanism might complement the PKA-mediated phosphorylation of RhoA described at the beginning of this Review, which enhances its interaction with RhoGDI. Interestingly, PAK1 also phosphorylates RhoGDI1 on Ser174 (as well as on Ser101), but this event inhibits its interaction with RAC1 (REF. 124). As both of these Ser residues are located in the hydrophobic pocket of the RhoGDI that accommodates the Rho GTPase geranylgeranyl group[119], it is possible that the phosphorylation of RhoGDI1 on Ser101 by PAK1 is what drives dissociation of the complex.

In addition to phosphorylation, RhoGDI1 undergoes sumoylation (TABLE 2). This increases its interaction with RAC1 (REF. 127), which subsequently reduces RAC1 and RhoA activity and decreases cancer cell migration and anchorage-independent growth[127,128]. As sumoylation is reversible, this mechanism would be a dynamic method of regulating RhoGDI function. XIAP, discussed earlier as an E3 ubiquitin ligase for RAC1, forms a complex with RhoGDI1 via its RING domain and thereby inhibits sumoylation[127,129]. It is therefore possible that RhoGDI1 brings XIAP and RAC1 together to promote RAC1 degradation.

RING domain
A Cys-rich tandem zinc-finger domain of 40–60 amino acids, often found in E3 ubiquitin ligases.

Protein interactions affect RhoGDI function. RhoGDI–Rho GTPase complexes can be regulated by specific protein interactions. Generally, proteins that bind to RhoGDIs prevent the formation of a Rho–RhoGDI complex. 14-3-3 proteins have been shown to function as pseudo-GDI proteins in the context of RND3 signalling[38], but they can also bind directly to Ser174-phosphorylated RhoGDIs and sequester them away from free Rho proteins[130]. It is unclear how this mechanism links with the apparent increased binding of Ser174-phosphorylated RhoGDI1 to RhoA (see above).

The regulation of protein interactions with RhoGDIs seems to be particularly important in neurons. For instance, the interaction of FERM domain-containing protein 7 (FRMD7) with RhoGDI1 initiates RAC1 signalling by releasing RAC1 from RhoGDI1 (REF. 131). Two mis-sense mutations in FRMD7 are known to reduce its ability to bind to RhoGDI and release RAC1, and a similar mechanism could account for the decreased RAC1 signalling and abnormal neuronal outgrowth seen in idiopathic congenital nystagmus, an X-linked disorder associated with FRMD7 (REF. 131). Likewise, TROY is a transmembrane receptor that interacts with the NOGO receptor complex to inhibit neurite outgrowth, for example, during axonal regrowth *in vivo*[132]. TROY binds to RhoGDI1 and thereby allows the release and subsequent activation of RhoA and inhibition of neurite growth[132]. It would be interesting to know whether FRMD7 and TROY selectively affect RhoGDI1 binding to RAC1 and RhoA, respectively, or whether they inhibit the binding of RhoGDI1 to all of its Rho GTPase targets.

Conclusions and future perspectives

It is becoming increasingly clear that signalling by Rho GTPases is regulated by a diverse range of mechanisms that go beyond classical GTP–GDP cycling. The high numbers of RhoGEFs and RhoGAPs compared to Rho GTPases indicate that particular cellular processes are likely to be controlled by specific regulators; however, these numbers alone cannot account for the wide range of roles that Rho GTPases carry out inside cells. An array of PTMs, as described here, is therefore needed to orchestrate the precise spatiotemporal activation of Rho GTPases required to accomplish these functions. These regulatory events are likely to be context-dependent and will vary among cell types and in response to different signals.

It is tempting to speculate that different cells might achieve specific localization patterns of GEFs and GAPs by differentially expressing scaffolding molecules and/or enzymes that carry out PTMs. This approach would allow the cell to coordinate the localization of Rho GTPases very quickly during dynamic processes, as well as to fine-tune Rho GTPase activities in tissue-specific contexts. Scaffolding complexes might be very important in determining not just the localization of Rho GTPases, but also their ability to undergo crosstalk. We envisage that these interactions will be a key area of research in the future, aided by advances in systems biology and proteomics.

Our understanding of the network of Rho GTPase regulation is far from complete. It is likely that effector proteins that regulate antagonistic or similar processes will also crosstalk with each other, as well as with RhoGEFs, RhoGAPs and RhoGDIs, during processes such as cell migration. It is also becoming apparent that effector proteins can provide positive or negative feedback to the appropriate Rho GTPases during cellular responses[38,133]. Protein complexes and protein modifications are highly dynamic, and thus it will be essential to follow these processes in real time in individual cells to determine how Rho GTPase activity is coordinated on a nanoscale level, for example, using super-resolution microscopy and Förster resonance energy transfer (FRET). Overall, the combination of GTP–GDP cycling and PTMs on Rho GTPases and their interacting partners provides multiple layers of regulation, which we propose have evolved to ensure that each Rho protein is activated at the right time and in the right place to enable cells in multicellular organisms to respond to their complex environments.

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Competing interests statement
The authors declare no competing interests.

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