Rac1 meets ubiquitin: New insights in Rac1 signalling
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Chapter 2

The role of ubiquitylation and degradation in RhoGTPase signalling

Micha Nethe and Peter L. Hordijk

Summary
Rho-like guanosine triphosphatases (GTPases) control many aspects of cellular physiology through their effects on the actin cytoskeleton as well as on gene transcription. Signalling by RhoGTPases occurs in a manner that is tightly coordinated and requires a series of regulatory proteins including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs and GAPs regulate GTPase cycling between the active (GTP-bound) and inactive (GDP-bound) state, whereas the GDI is a cytosolic chaperone that binds inactive RhoGTPases.

Like many other proteins, RhoGTPases are subject to degradation following the covalent conjugation of ubiquitin. There have been increasing indications that ubiquitylation of small GTPases occurs in a regulated fashion, primarily upon activation, and is an important means to control signalling output. Recent work has identified cellular proteins that control Ras and RhoGTPase ubiquitylation and degradation and thus allows us to amend the canonical model for GTPase (in)activation. Moreover, accumulating evidence for indirect regulation of GTPase function through the ubiquitylation of GTPase regulators makes this post-translational modification a key feature of GTPase-dependent signalling pathways. Here we will discuss these recent insights in the regulation of RhoGTPase ubiquitylation and their relevance for cell signalling.

Introduction
RhoGTPases are members of the Ras GTPase superfamily and are key regulators of the cellular cytoskeleton. They control cell adhesion, migration, gene transcription and cell division (Bosco et al., 2009; Didsbury et al., 1989; Hall, 1990; Kozma et al., 1997; Ridley et al., 1992; Ridley and Hall, 1992). Although the 22 different RhoGTPases show very high sequence homology, they have unique biological effects (Bishop and Hall, 2000; Bosco et al., 2009; van Aelst and D'Souza-Schorey, 1997). In polarized, migrating cells, RhoA stimulates myosin-based contractility of the actin cytoskeleton, which drives retraction of the rear of the cell (Alblas et al.,
Cdc42 and Rac1 promote actin polymerization resulting in formation of either lamellapodia (Rac1) or filopodia (Cdc42) via activation of the Arp2/3 complex, which drives cell protrusion at the leading edge of a migrating cell (Insall and Machesky, 2009).

Most RhoGTPases act as molecular switches, cycling between a GDP- and a GTP-bound state (Fig. 1) (Bishop and Hall, 2000; Bosco et al., 2009; Rossman et al., 2005; van Aelst and D’Souza-Schorey, 1997). Binding of GTP induces a conformational change which allows the binding and subsequent activation of effector proteins (Didsbury et al., 1989; Hall, 1990; Kozma et al., 1997; Ridley et al., 1992; Ridley and Hall, 1992). Intrinsic GTP hydrolysis then reverts the GTPase to its inactive GDP-bound conformation (Didsbury et al., 1989; Hall, 1990; Kozma et al., 1997; Ridley et al., 1992; Ridley and Hall, 1992). GEFs catalyse the exchange of GDP for GTP, thus activating the RhoGTPase. In contrast, GAPs promote the intrinsic GTPase activity. Finally, RhoGTPases can associate with cytosolic chaperone proteins, the GDIs, which maintain the GTPase in its inactive conformation (Didsbury et al., 1989; Hall, 1990).

Although the GDP-bound form is generally considered to be inactive, GDP-bound RhoGTPases can nevertheless exert signalling functions. Rac1, in complex with Rho-GDI, can activate the NADPH oxidase (NOX) complex (Grizot et al., 2001). Similarly, binding of RhoGDI does not prevent the Rac1-, or Cdc42-driven activation of phospholipase C-β2 (Illenberger et al., 1998). Likewise, RhoB, which regulates vesicle traffic (Fernandez-Borja et al., 2005; Neel et al., 2007; Wherlock et
al., 2004), controls endosomal sorting both in the GDP- or GTP-bound form (Neel et al., 2007).

These findings indicate that GDP-bound RhoGTPases are signalling competent and suggest that mechanisms, other than GTP hydrolysis, must exist to terminate RhoGTPase signalling. Recently, conjugation to ubiquitin and consequently proteasomal degradation has been shown to regulate signalling by RhoGTPases, such as RhoA and Rac1 (Chen et al., 2009; Kovacic et al., 2001; Lerm et al., 2002; Lynch et al., 2006; Nethe et al., 2010; Visvikis et al., 2008; Wang et al., 2003). Here, we will review recent data on the ubiquitylation of RhoGTPases that support the notion that this post-translational modification is an important aspect of GTPase regulation and signalling.

**Protein ubiquitylation in cell signalling**

Protein ubiquitylation represents a three-step process resulting in the covalent attachment of ubiquitin, a 76 amino acid protein, to lysine residues within target proteins. Ubiquitylation is initiated by an ubiquitin-activating enzyme (E1) which drives ATP-dependent transfer of ubiquitin to an ubiquitin-conjugating enzyme (E2) that, in conjunction with an ubiquitin protein (E3) ligase, covalently attaches the ubiquitin to the target. There are several hundreds of proteins that, based on established activity or structural features, could serve as potential E3 ligases (Deshaies and Joazeiro, 2009). These can be divided in two superfamilies, the RING (really interesting new gene) E3 ligases and the HECT (homologous to the E6-AP carboxyl terminus) E3 ligases (see Box 1) (Rotin and Kumar, 2009).

A conjugated ubiquitin can serve as a new target for (poly-) ubiquitylation. Whereas it is generally accepted that mono-ubiquitylation drives protein internalisation from the plasma membrane, poly-ubiquitylation serves primarily as a signal for proteasomal degradation, which, in addition to the lysosomal pathway, controls protein turnover. Ubiquitin contains within its sequence seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) that can be used to form various types of ubiquitin-chains. K48-linked ubiquitylation is associated with proteasomal degradation, in contrast to K63-linked ubiquitylation, which plays a role in
regulating protein traffic as well as in DNA repair (Acconcia et al., 2009; Welchman et al., 2005; Xu et al., 2009). Ubiquitylation might also affect protein-protein interactions, enzymatic activity and subcellular localisation (Acconcia et al., 2009; Haglund et al., 2003; Holler and Dikic, 2004; Welchman et al., 2005). Conjugation by ubiquitin allows binding to proteins containing a ubiquitin-recognition motif such as the endocytic proteins Eps15 and Hrs (Polo et al., 2002). In addition, ubiquitin can also associate with a subset of SH3 domains, regions of ~60 amino acids that mediate protein-protein interactions (Stamenova et al., 2007). Ubiquitylation is reversible, as the ubiquitin moiety can be removed by Ubiquitin-Specific Proteases (USPs) (Sowa et al., 2009).

In summary, ubiquitin conjugation is a bona fide signalling event due to its regulation of protein localisation, protein-protein interactions and effects on expression levels. Ubiquitylation is also relevant for signalling by and regulation of the RhoGTPases, as discussed below (Chen et al., 2009; Kovacic et al., 2001; Lerm et al., 2002; Lynch et al., 2006; Nethe et al., 2010; Visvikis et al., 2008; Wang et al., 2003).

The role of ubiquitylation in RhoGTPase signalling
Over the past two decades, Rac1, RhoA and Cdc42 have become the most extensively studied members of the RhoGTPase family. In line with this, information on GTPase ubiquitylation has been obtained primarily for these proteins (Table 1). In this section, we will discuss the available information in more detail, underscoring the relevance of ubiquitylation for RhoGTPase regulation and signalling and supporting the notion that ubiquitylation represents an additional means of crosstalk between different RhoGTPases.

Rac GTPases
The first evidence for proteasome-mediated downregulation of the Rac GTPases came from analysis of Rac1-stimulated activation of NOXs, that lead to the production of reactive oxygen species (ROS) (Kovacic et al., 2001). Inhibition of NOX activity revealed an unexpected proteasome-dependent increase of ectopically
expressed, active Rac1 (V12) protein expression, but not of inactive Rac1 (N17) (Kovacic et al., 2001). As Rac1 acts upstream of NOX, this suggests that the production of ROS triggers a proteasome-dependent negative feedback loop that mediates Rac1 degradation and thus blocks Rac1 signalling.

Inhibition of Rac1 by ubiquitin-proteasome system (UPS)-mediated degradation also occurs during the onset of epithelial-mesenchymal transition (EMT) (Lynch et al., 2006). EMT is marked by the disassembly of cell-cell contacts and increased cell motility (Perez-Moreno et al., 2003). Since Rac1 activation stimulates the formation of epithelial junctions, its activity needs to be down-regulated during EMT (Hordijk et al., 1997; Palamidessi et al., 2008) and a transient decrease of Rac1 activity was indeed shown in Madin-Darbey Canine Kidney (MDCK) cells. Unexpectedly, this decrease was accompanied by a reduction in Rac1 protein levels, in addition to Rac1 inactivation (Lynch et al., 2006). Inhibition of the proteasome impaired the HGF-induced decrease of Rac1 protein levels and inhibited EMT. This indicates that during EMT, Rac1 signalling is silenced by UPS-mediated degradation, either in addition to or instead of its inactivation by a RacGAP. This notion is further supported by our own studies on the regulation of Rac1 levels by the membrane-associated adapter Caveolin-1 (Cav1) (Nethe et al., 2010, see below).

The cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* has been an important tool in the analysis of Rac1 degradation. CNF1 deaminates Rac1 at glutamine 61, which results in constitutive association of Rac1 to GTP, activating Rac1 (Lerm et al., 1999). Activation by CNF1 induces Rac1 ubiquitylation at lysine residue 147 and its subsequent proteasomal degradation (Boyer et al., 2006; Doye et al., 2002; Lerm et al., 1999; Visvikis et al., 2008). In addition to lysine 147, the polybasic, hypervariable region at the C-terminal end of Rac1 is also involved in regulating its degradation (Lanning et al., 2004; Pop et al., 2004). When this region in Rac1 is replaced by that present in Rac2 or Rac3, its CNF1-induced degradation is inhibited, indicating that this domain is specifically required for Rac1 degradation (Pop et al., 2004). The Rac1 C-terminus mediates specific protein-protein interactions such as with the RacGEF β-PIX, Crk and CD2-associated protein
(Nethe et al., 2010; ten Klooster et al., 2006; van Duijn et al., 2010; van Henrik et al., 2003; Williams, 2003), and our laboratory showed that this region also binds to Cav1, an important regulator of protein internalization and of a large number of cell signalling pathways. We further showed that Cav1 regulates Rac1 ubiquitylation and degradation (Nethe et al., 2010). Loss of Cav1 induces accumulation of non-ubiquitylated Rac1 as well as mono-ubiquitylated Rac1, which indicates that Cav1 selectively regulates the degradation of poly-ubiquitylated activated Rac1 (Nethe et al., 2010). These findings further suggest that mono-ubiquitylated Rac1 might have a distinct biological function as a result of its differential localization and association to other regulatory proteins.

It is currently unclear at which subcellular location Rac1 ubiquitylation and degradation occurs. Cav1 has been implicated in integrin-dependent internalization of Rac1-containing membrane domains, which is accompanied by a loss of interactions between Rac1-and its effectors and inhibition of Rac1-mediated signalling (del Pozo et al., 2004). As loss of Cav1 does not impair Rac1 mono-ubiquitylation, this step might occur at the plasma membrane. Similar to what has been described for Ras (see Box 2), we found that an N-terminally linked ubiquitin-Rac1 fusion construct, used to mimic mono-ubiquitylated Rac1, localizes prominently to endosomal structures, rather than to the plasma membrane (Fig. 2). In addition, a K147R mutant of activated Rac1, which cannot be ubiquitylated, shows an enhanced accumulation at the plasma membrane (Nethe et al., 2010).

Fig 2. Fusion of ubiquitin with Rac1 relocates Rac1 towards endocytic vesicles. Imaging of Rac1-Q61LK147R and a Rac1 N-terminal fusion construct with ubiquitin (Ub-Rac1-Q61LK147R) by confocal microscopy in fixed Hela cells showed a significant accumulation of the Ub-Rac1 fusion at endosomal structures indicated by the arrows (Bar, 10μm). This localization is in marked contrast to the active Rac1Q61LK147R construct that can not be ubiquitylated and which localises predominantly at the plasma membrane, indicating that mono-ubiquitylation of Rac1 can regulate Rac1 subcellular targeting.
Thus, it is tempting to speculate that (mono)-ubiquitylation of Rac1 drives its internalisation in a Cav1-dependent fashion. Several studies reported that proteasomal degradation of poly-ubiquitylated Rac1 occurs in the nucleus (Esufali et al., 2007; Lanning et al., 2004; Sandrock et al., 2009). Proteasomal degradation of Rac1 was inhibited when the nuclear localisation signal (NLS) found within the Rac1 C-terminus is mutated, (Lanning et al., 2004; Sandrock et al., 2009) or when Karyopherin-α2, a nuclear import factor which has also been implicated in the nuclear translocation of Rac1, is silenced (Lanning et al., 2004; Sandrock et al., 2009). Moreover, inhibition of Wnt signalling within the nucleus correlates with an increase in K48-linked poly-ubiquitylation and stabilization of active Rac1 in the nucleus (Esufali et al., 2007). These data indicate that Rac1 localization and thus its degradation is regulated by Cav1 and by Karyopherin-α2 (Fig. 3).

![Fig 3. Schematic overview of Rac1 regulation by ubiquitylation.](image)

Active Rac1 recruits Cav1 towards Focal Adhesions (FAs), clusters of ligand-bound integrins and -associated proteins that concentrate at the end of F-actin stress fibers. Subsequent mono-ubiquitylation of Rac1 stimulates Rac1 internalisation in a Cav1-dependent fashion. Mono-ubiquitylated Rac1 can either be de-ubiquitylated by members of the USP (Ubiquitin-Specific Protease) family or be poly-ubiquitylated followed by proteasomal degradation. Karyopherin-α2 controls the poly-ubiquitylation and proteasomal degradation of Rac1 in the nucleus and could therefore be required for the translocation of mono-ubiquitylated Rac1 into the nucleus.

The E3 ubiquitin ligase(s) that target(s) Rac1 are unknown, as is the subcellular location of Rac1 ubiquitylation. Rac1 associates with several ubiquitin ligases, including the RING-finger ligase plenty of SH3s (POSH) (Kim et al., 2006; Visvikis et al., 2008), Cbl (Cas-Br-M (murine) ecotropic retroviral transforming sequence) (Sattler et al., 2002; Schmidt et al., 2006; Teckchandani et al., 2005) and the SCF(βTrCP)-E3 ligase complex (Boyer et al., 2004; Senadheera et al., 2001). However none of these E3 ubiquitin ligases were shown to target Rac1 for ubiquitylation (Senadheera et al., 2001; Visvikis et al., 2008). Thus, the
identification of the ubiquitin ligase for Rac1 remains a key objective for future research in this area.

**Cdc42**

The RhoGTPase Cdc42 is best known for its induction of actin polymerization and formation of filopodia, finger-like membrane protrusions. Cdc42 is, similar to RhoA and Rac1, susceptible to CNF1-mediated degradation (Doye et al., 2002), but the mechanism by which Cdc42 is targeted for ubiquitylation remains to be elucidated. Interestingly, Cdc42 prevents the proteasomal degradation of the epidermal growth factor (EGF) receptor by sequestering the Ring E3 ligase Cbl (Feng et al., 2006; Hirsch et al., 2006; Wu et al., 2003). However, Cbl does not target Cdc42 for ubiquitylation, but regulates the ubiquitylation and degradation of the Cdc42/Rac1 GEF β-Pix (Schmidt et al., 2006) (Fig 4A). This is part of a negative feedback mechanism for EGF-induced signalling, as inactive Cdc42 fails to sequester Cbl, which allows Cbl-mediated degradation of the EGF receptor. For Cdc42, inhibition of its signalling by targeting a GEF appears an important pathway, as Smurf-1 can ubiquitylate the Cdc42 GEF hPEM-2 but not Cdc42 itself (Yamaguchi et al., 2008) (Fig 4A). This suggests that Smurf-1, upon its recruitment by PKCζ into the Par6-Cdc42 polarity complex, facilitates the degradation of active RhoA and also forms part of a negative feedback loop by inhibiting hPEM-2-mediated activation of Cdc42. Similarly, another E3 ligase Cullin-1, which exists in a complex with Skp1 and Rbx-1 inhibits Cdc42 activation by targeting its GEFs FGD1 and FGD3 for ubiquitylation (Hayakawa et al., 2005; Hayakawa et al., 2008). Thus, while the exact underlying mechanisms of UPS targeting of Cdc42 remain to be elucidated, a growing number of studies indicate that the UPS controls Cdc42 through regulating the available pool of Cdc42 GEFs (Fig. 4A).

**RhoA**

UPS targeting and degradation of RhoA was first observed following CNF1-stimulated activation of RhoA, analogous to its regulation of Rac1 (Doye et al., 2002; Lerm et al., 2002; Schmidt et al., 1997). CNF1 induces proteasomal
degradation of activated RhoA in different cell types, including 804G and HEK293 epithelial cells, NIH-3T3 fibroblasts, human endothelial cells, primary fibroblasts and macrophages (Boyer et al., 2006; Doye et al., 2002). In this respect, RhoA is regulated very differently compared to its close relative RhoB, which is primarily degraded via the lysosomal pathway (Adamson et al., 1992; Perez-Sala et al., 2009; Stamatakis et al., 2002).

Smurf-1 (smad ubiquitin regulatory factor-1), initially identified to control TGF-β signalling by targeting the SMAD family of transcriptional regulators for proteasomal degradation (Zhu et al., 1999), was the first E3 ligase found to target RhoA for ubiquitylation (Wang et al., 2003). Smurf-1 induces membrane protrusion, loss of actin stress fibres and reduced cell motility in Mv1Lu epithelial, HEK-3T3 cells and MDAMB-231 breast cancer cells and induces enhanced neurite outgrowth in Neuro2a neuroblastoma cells, suggestive for inhibition of RhoA signalling (Bryan et al., 2005; Sahai et al., 2007; Wang et al., 2003). Smurf-1 appears to be RhoA-specific, as it ubiquitylates RhoA, but not Rac1 or Cdc42, and, moreover, Smurf-2, a Smurf-1 homologue, is incapable of ubiquitylating RhoA (Wang et al., 2003). Smurf-2 might even counteract Smurf-1, as it can induce Smurf-1 ubiquitylation and degradation (Fukunaga et al., 2008). Ubiquitylation by Smurf-1 is limited to activated RhoA, since genetic loss of Smurf-1 blocked proteasomal-degradation of CNF-1 activated RhoA in fibroblasts (Boyer et al., 2006) (Fig. 4B). In agreement with this, protein kinase A (PKA)-induced phosphorylation of RhoA at Serine 188, which enhances RhoA inactivation through its association with Rho GDI, significantly impairs Smurf-1-mediated degradation of RhoA (Rolli-Derkinderen et al., 2005).

Interestingly, Smurf-1 ubiquitylation of RhoA is regulated by atypical protein kinase C zeta (PKCζ), and PKCζ binds to and co-localises with Smurf-1 at membrane protrusions (Wang et al., 2003). PKCζ is a key component of the PAR6-Cdc42 polarity complex (Joberty et al., 2000; Suzuki et al., 2001; Yamanaka et al., 2001), which localizes at the leading edge in migrating cells (Etienne-Manneville and Hall, 2001). PKCζ has been proposed to target Smurf-1 to the PAR6-Cdc42 polarity complex, thereby stimulating a local down-regulation of RhoA signalling at
the leading edge to promote cell motility (Sahai et al., 2007; Zhang et al., 2004) (Fig. 4B). In line with this model, Smurf-1 was found to associate with phosphorylated PAR6 following TGFβ stimulation of murine mammary gland cells, which results in local degradation of RhoA, necessary to dissolve tight-junctions during EMT (Ozdamar et al., 2005).

A second E3 ligase for RhoA is Cullin-3, which does not bear any resemblance to Smurf-1 (Chen et al., 2009). Cullin-3 belongs to the class of cullin proteins that function as scaffolds in multi-protein Cullin-Ring Ligases (CRLs) (Petroski and Deshaies, 2005). HeLa cells depleted of Cullin-3 show a remarkable increase in actin stress fiber formation, which was associated with an increase in RhoA protein levels. In line with this, Cullin-3 only targets GDP-bound RhoA for ubiquitylation, but not RhoB, RhoC, Cdc42 or Rac1 (Chen et al., 2009) (Fig. 4B). Thus, next to its GTPase specificity, Cullin-3 stands out as it regulates inactive rather than active RhoA. Intriguingly, loss of Cullin-3 induces an increase in both the total pool of RhoA as well as of active, GTP-bound RhoA (Chen et al., 2009). Whether this is a consequence of an increase in the amount of RhoA protein that is available for activation, or whether it points to a role of Cullin-3 in regulating the balance between RhoA GEFs and GAPs remains to be established.

Cullin-3 recruits BTB (bric-à-brac, tramtrack, broad-complex) proteins which act as scaffolds, recruiting substrates for ubiquitylation by CRLs (Petroski and Deshaies, 2005). Chen and colleagues (Chen et al., 2009) identified a BTB protein encoded by the gene CG10465 that facilitates RhoA targeting by cullin-3 and they named this protein BACURD for BTB-containing adaptor for Cullin-3 mediated RhoA degradation. The physiological relevance of Cullin-3 and BACURD mediated degradation of RhoA is further underscored by the observation that depletion of Cullin-3 or of BACURD impairs cell movement in vitro and in vivo (Chen et al., 2009).

The identification of Smurf-1 and Cullin-3 E3 ligases, which do not show any structural homology and also target different forms of RhoA, underscores the complexity of UPS-targeting of RhoA (Fig. 4B). As different E3 ligases can target RhoA for proteasomal degradation, it is thus tempting to speculate that, analogous to
the role of different GEFs and GAPs in driving the spatio-temporal (in)activation of RhoA, the UPS might employ distinct E3 ligases to control the spatio-temporal degradation of RhoA.

*Rho bric-à-brac, tramtrack, broad-complex-2 (RhoBTB-2)*

A number of RhoGTPases such as RhoE and RhoH exhibit a rather poor intrinsic GTPase hydrolysis and do not appear to be regulated by GEFs or GAPs. Insights into the regulation by the UPS of these atypical RhoGTPases (Aspenstrom et al., 2007; Chardin et al., 1993) are currently limited, but the association of the atypical RhoGTPase RhoBTB2 with the ring ligase Cullin-3 (Aspenstrom et al., 2007; Wilkins et al., 2004) is of interest. The RhoGTPase subfamily of RhoBTBs comprises three members (RhoBTB1-3), of which RhoBTB2, also known as DBC2 (Doubly deleted in breast cancer2), is the best characterised (Aspenstrom et al., 2007). RhoBTB2 associates with *Drosophila* PakB and with mammalian Cullin-3 (de la et al., 2005; Wilkins et al., 2004). Binding to Cullin-3 mediates RhoBTB2 ubiquitylation and leads to its downregulation in HeLa, 293T and SK-MES-1 cells (Wilkins et al., 2004) (Fig. 4C). Interestingly, reintroducing a RhoBTB2 mutant (Y284D) that is unable to bind Cullin-3 and therefore not susceptible to Cullin-3-mediated degradation, cannot compensate for the loss of RhoBTB2. As BTB-proteins, such as BACURD, mediate Cullin-3 target recognition (Chen et al., 2009), the failure of RhoBTB2(Y284D) to overcome the loss of RhoBTB2 suggests that RhoBTB2 acts as a scaffold protein and needs to associate with Cullin-3 to function. Because RhoBTB2 might be involved in determining Cullin-3 target specificity, it (Chen et al., 2009) could play a role in the regulation of RhoA by Cullin-3 and facilitating Cullin-3-dependent ubiquitylation of RhoA (Fig. 4C). Interestingly, breast, head and neck cancer show loss of RhoBTB2 protein and a concomitant increase of RhoA protein levels, which could lead to increased RhoA signalling (Abraham et al., 2001; Beder et al., 2006; Fritz et al., 1999; Hamaguchi et al., 2002).
Fig 4. Overview of the ubiquitin-mediated crosstalk between different RhoGTPases. (A) The association of Cbl with active Cdc42 stimulates a negative feedback loop by initiating the ubiquitylation and proteasomal mediated degradation of the Cdc42 GEF βpix. In addition, active Cdc42 itself is also susceptible to poly-ubiquitylation and proteasomal degradation, but the ubiquitin E3 ligases involved are unknown. (B) Recruitment of the HECT E3 ligase Smurf-1 by active Cdc42 in complex with PAR6 and PKCζ subsequently stimulates the local degradation of active RhoA. In parallel, this triggers a negative feedback loop as Smurf-1 poly-ubiquitylates the Cdc42 GEF hPEM-2, driving its proteasomal degradation. In addition, inactive GDP-bound RhoA is targeted by BACURD which promotes its Cullin-3-driven ubiquitylation and proteasomal degradation. (C) Similarly, Cullin-3 regulates the ubiquitylation and proteasomal degradation of RhoBTB2. Interestingly, RhoBTB2, like BACURD, contains a BTB region and may thus also bind to and act as a scaffold for Cullin-3, regulating degradation of Cullin3 substrates. As loss of the RhoBTB2 protein correlates with an increase in RhoA expression in several cancers, RhoBTB2 may well be involved in Cullin-3-driven ubiquitylation and proteasomal degradation of RhoA.

**Crosstalk between the UPS and RhoGTPases by RhoGDI**

There are three RhoGDIs genes in mammals: RhoGDIα, RhoGDIβ (also know as Ly-GDI or D4-GDI) and RhoGDIγ. Recent data suggest that GDIs act not only as chaperones for inactive GTPases, but also regulate the expression of RhoGTPase proteins (Boulter et al., 2010; Ho et al., 2008). Ho and co-workers found that siRNA-based silencing of RhoA and RhoC expression leads to the accumulation of RhoB protein in human adenocarcinoma cells, melanoma cells and primary human fibroblasts (Ho et al., 2008). As the number of RhoGDI molecules approximately equals the number of RhoGTPase molecules in the cell (Michaelson et al., 2001), reduced expression of RhoA and RhoC could result in an enhanced binding of RhoGDI to RhoB, thus protecting it from degradation. This hypothesis was supported by expression of RhoGDIα which substantially increased the stability of RhoB (Ho et al., 2008).
A recent study of Boulter and co-workers showed that siRNA-induced depletion of RhoGDIα was found to reduce the levels of RhoA, RhoC, Rac1 and Cdc42, but not RhoB (Boulter et al., 2010). In a complementary set of experiments, overexpression of RhoGTPases was found to reduce the stability and activity of endogenous Rho proteins as a result of competitive binding to RhoGDI (Boulter et al., 2010). These findings indicate that association of RhoGDIIs with RhoGTPases not only maintains them in an inactive state, but also protects them from degradation by the UPS. This is in good agreement with the general notion that activated GTPases are more susceptible to degradation (Jura et al., 2006; Kovacic et al., 2001; Schmidt et al., 1997; Visvikis et al., 2008). Finally, it is important to underscore the relevance of the analysis of endogenously expressed proteins in studies on ubiquitylation and protein stability. Although expression of GTPase mutants can be informative, the Boulter study shows that these may, indirectly, also affect the levels of the endogenous GTPases, potentially confounding experimental results.

Concluding remarks
It has been over two decades since the identification of RhoGTPase-based signalling (Bosco et al., 2009; Didsbury et al., 1989; Hall, 1990; Kozma et al., 1997; Ridley et al., 1992; Ridley and Hall, 1992). During this period, work on the large number of GEFs and GAPs and on the role of RhoGDI have led to a widely accepted model in which GDP- or GTP-binding is a direct measure for the inactive or active state of a RhoGTPase. However, it has been questioned whether GTP hydrolysis alone is sufficient to block RhoGTPase signalling (Grizot et al., 2001; Illenberger et al., 1998; Neel et al., 2007). The UPS likely represents a parallel mechanism for inactivating RhoGTPases by targeting them for degradation. The more we appreciate the complexity of RhoGTPase regulation in time and space (Pertz, 2010), the more it becomes it is likely that additional mechanisms such as the UPS discussed here play a role through controlled degradation of either activated RhoGTPases or their regulators. In addition, ubiquitylation of RhoGTPases might mediate additional yet unidentified protein interactions and so modify their subcellular localisation, further complicating the canonical cycling model (Fig 1).
Novel techniques, such as the FRET-based detection of ubiquitylation (Batters et al., 2010; Ganesan et al., 2006) will be important to allow visualisation of protein-ubiquitin conjugation in live cells. Identification of the relevant E3 ligases at play, their target lysine residues, the type of ubiquitylation and its functional consequences are therefore obvious goals for future research, making analysis of RhoGTPase signalling all the more fascinating.

Table 1. Ubiquitylation of RhoGTPases.

<table>
<thead>
<tr>
<th>RhoGTPases</th>
<th>Ubiquitylation</th>
<th>Position</th>
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<tr>
<td>Rac1</td>
<td>mono-, poly</td>
<td>K147</td>
<td></td>
<td>(Doye et al., 2002; Esufali et al., 2007; Kovacic et al., 2001; Lanning et al., 2004; Lerm et al., 2002; Nethe et al., 2010; Sandrock et al., 2009; Visvikis et al., 2008)</td>
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<tr>
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<td>poly</td>
<td></td>
<td></td>
<td>(Visvikis et al., 2008)</td>
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<tr>
<td>Cdc42</td>
<td>poly</td>
<td></td>
<td></td>
<td>(Doye et al., 2002)</td>
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<tr>
<td>RhoA</td>
<td>poly</td>
<td>K6, K7</td>
<td>Smurf-1, Cullin-3</td>
<td>(Boyer et al., 2006; Bryan et al., 2005; Chen et al., 2009; Doye et al., 2002; Ozdamar et al., 2005; Rolli-Derkinderen et al., 2005; Sahai et al., 2007; Wang et al., 2003; Wang et al., 2006; Zhang et al., 2004)</td>
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<tr>
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<td></td>
<td>(Perez-Sala et al., 2009; Stamatakis et al., 2002)</td>
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<td>RhoBTB2</td>
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<td></td>
<td>Cullin-3</td>
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Box 1. RING vs HECT: Two superfamilies of E3 ligases. The E3 ligases can be divided into two superfamilies, the RING E3 ligases and the HECT E3 ligases (Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009). The conserved HECT domain comprises ~350 amino acids and was first identified in human papilloma virus E6-associated protein (Huibregtse et al., 1995). Based on their N-terminal domains, the 28 identified human HECT E3 ligases are classified as either Nedd4- or HERC- (HECT and RLD domain) family proteins or as the unrelated HECT E3 ligases. In contrast to the small HECT superfamily, 616 human genes have been identified to encode a RING motif, exceeding significantly the number of HECT E3 ligases (Li et al., 2008). The RING domain is characterised by conserved cysteine and histidine residues that maintain the RING three-dimensional structures by supporting the binding of two zinc atoms. Numerous RING variants have been reported to exist in multi-protein complexes, as exemplified by the class of cullin-RING ubiquitin ligases (CRL), these ligases comprises a cullin isoform as well as associated subunits involved in stabilising the RING structure and driving target recognition (Deshaies and Joazeiro, 2009). RING and HECT E3 ligases can furthermore be distinguished by the way that they transfer ubiquitin to a substrate. The HECT domain contains a conserved catalytic cysteine, which, upon association with E2 enzymes carrying the ubiquitin, initiates a thio-ester bond with the ubiquitin C-terminus. This facilitates the subsequent transfer of ubiquitin to a substrate. In contrast, RING E3 Ligases act as a platform that scaffolds E2 enzymes and subsequent substrates, catalysing the direct transfer of ubiquitin from the E2 enzyme to the substrate as illustrated in the Box Figure.
Box 2. Ubiquitylation regulates Ras signalling

Regulation of Ras signalling, like activation and its sub-cellular targeting, comprises many similarities with that of RhoGTPases (Fehrenbacher et al., 2009). The proto-oncogenic Ras GTPases H- and N-Ras are subject to mono- and K63-linked di- ubiquitylation (Jura et al., 2006), which acts as a regulatory signal that triggers the internalization of membrane-bound proteins towards endocytic compartments (Acconcia et al., 2009; Welchman et al., 2005). Because ubiquitylation of Ras is initiated by its activation and requires farnesylation or palmitoylation, Ras ubiquitylation likely occurs at the plasma membrane (Jura et al., 2006). Since endosomes are derived from internalised plasma membrane domains, ubiquitylation of Ras could provide a molecular mechanism that drives the internalisation of activated N- and H-Ras (Box Figure). The removal of activated Ras from the plasma membrane suggests that ubiquitylation serves to downregulate Ras signalling. In agreement with this notion, ubiquitylation of Ras prevents uncontrolled Ras activation in Drosophila (Yan et al., 2009). Recently, the βTrCP RING E3 ligase was found to drive poly-ubiquitylation and subsequent proteasomal degradation of H-Ras in HEK293 cells (Kim et al., 2009) (Box Figure). This pathway is reminiscent of the βTrCP-mediated down-regulation of β-catenin (Kim et al., 2009). Mutations in β-catenin have been implicated in the most common human malignant tumor, hepatocellular carcinoma (HCC) (Harada et al., 2004). Although mutations leading to constitutive activation of H-Ras are not sufficient to induce HCC, activating mutations in both β-catenin and H-Ras cause a 100% incidence of HCC in mice (Harada et al., 2004). Thus, βTrCP-driven downregulation of Ras could act as a safety mechanism to avoid excessive activation of Ras during Wnt/β-catenin signalling, preventing tumor development. Taken together, the ubiquitylation, internalisation and downregulation of activated Ras might serve, in
addition to RasGAP-stimulated GTP hydrolysis, to control the extent of Ras signalling.

References


