Review

Special Issue – 3D Cell Biology

Rho protein crosstalk: another social network?

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Many fundamental processes in cell biology are regulated by Rho GTPases, including cell adhesion, migration and differentiation. While regulating cellular functions, members of the Rho protein family cooperate or antagonize each other. The resulting molecular network exhibits many levels of interaction dynamically regulated in time and space. In the first part of this review we describe the main mechanisms of this crosstalk, which can occur at three different levels of the pathway: (i) through regulation of activity, (ii) through regulation of protein expression and stability, and (iii) through regulation of downstream signaling pathways. In the second part we illustrate the importance of Rho protein crosstalk with two examples: integrin-based adhesion and cell migration.

Connecting Rho family members

All eukaryotic cells contain Rho GTPases (ranging from six in yeast to ~20 in mammals) and they are implicated in the regulation of many biological processes, from adhesion and motility to gene expression and differentiation [1]. As a consequence of their biological ubiquity, Rho proteins often cooperate or antagonize each other to control cellular tasks. This interaction between Rho family members relies on a complex molecular dialog taking place at different levels in their signaling pathways. The first observation of an interaction between two Rho proteins was made by Ridley *et al.* in 1992. In their seminal paper they showed that ruffle formation in growth factor-stimulated fibroblasts was due to Rac1 and that this led to stress fiber formation in a RhoA-dependent manner [2]. Since then an amazing variety of mechanisms have been described that interconnect the members of the Rho family.

Cycling between an inactive GDP state and an active GTP state, Rho proteins are usually compared to molecular switches. Three classes of proteins regulate their cycle: guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs) [1]. GEFs activate Rho proteins by catalyzing the exchange of GDP for GTP [3], whereas GAPs stimulate the intrinsic GTPase activity and promote the return to the inactive state [4]. The inactive pool of Rho proteins is maintained in the cytosol by association with GDI. In the active GTP-bound conformation they interact with effectors and perform their functions. The reader is directed to recent comprehensive reviews for information about Rho protein regulation, Rho GEFs, GAPs, GDI and effectors [1,3–6]. We focus here on the pathways and proteins that connect Rho proteins with each other. After discussing several specific mechanisms we illustrate the importance of these interactions with two examples, integrin-based cell adhesion and cell migration, in which coordination between Rho proteins is essential.

Molecular mechanisms of Rho protein crosstalk

Different modes of interaction between Rho GTPases are illustrated in Figure 1. There are three main levels at which Rho family members interact: (i) regulation of activity (i.e. via a GEF or a GAP); (ii) regulation of protein expression and stability, in which RhoGDI is important; and (iii) regulation of downstream signaling pathways.

Crosstalk via GEFs and GAPs

The quintessential interaction between Rho proteins is illustrated by RhoA and Rac1, two ubiquitous and well-studied family members. Selective activation of one Rho protein is easily achieved when a signaling pathway acts on a GEF with a single specificity. However, many GEFs (e.g. Vav2) can activate multiple Rho proteins, including both RhoA and Rac1. There may be pathways where both proteins are simultaneously activated, but in many situations the activation of RhoA and Rac1 appears to be separated either temporally or spatially, or one of the proteins is activated and the other inhibited. There are several examples where RhoA and Rac1 modulate each other through regulation of GEFs and GAPs (Figure 2, Table 1). Although Rac1 was originally identified as stimulating RhoA activity, in most situations these two proteins exhibit an antagonistic relationship that operates at multiple levels. This opposition can be reciprocal or unidirectional, as was observed in a classic study in which Rac1 activation in NIH3T3 cells induced an epithelial morphology, including cadherin-based junctions, and was accompanied by decreased RhoA activity [7]. Elevated RhoA activity reversed the phenotype, promoting a mesenchymal fibroblastic morphology, but did not inhibit Rac1 activity [7].

RhoA inhibition of Rac1

Inhibiting the RhoA effector Rho-associated kinase (ROCK) induces membrane protrusions at random positions around the cell periphery, and this was observed to be

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 0962-8924/$ – see front matter doi:10.1016/j.tcb.2011.08.002

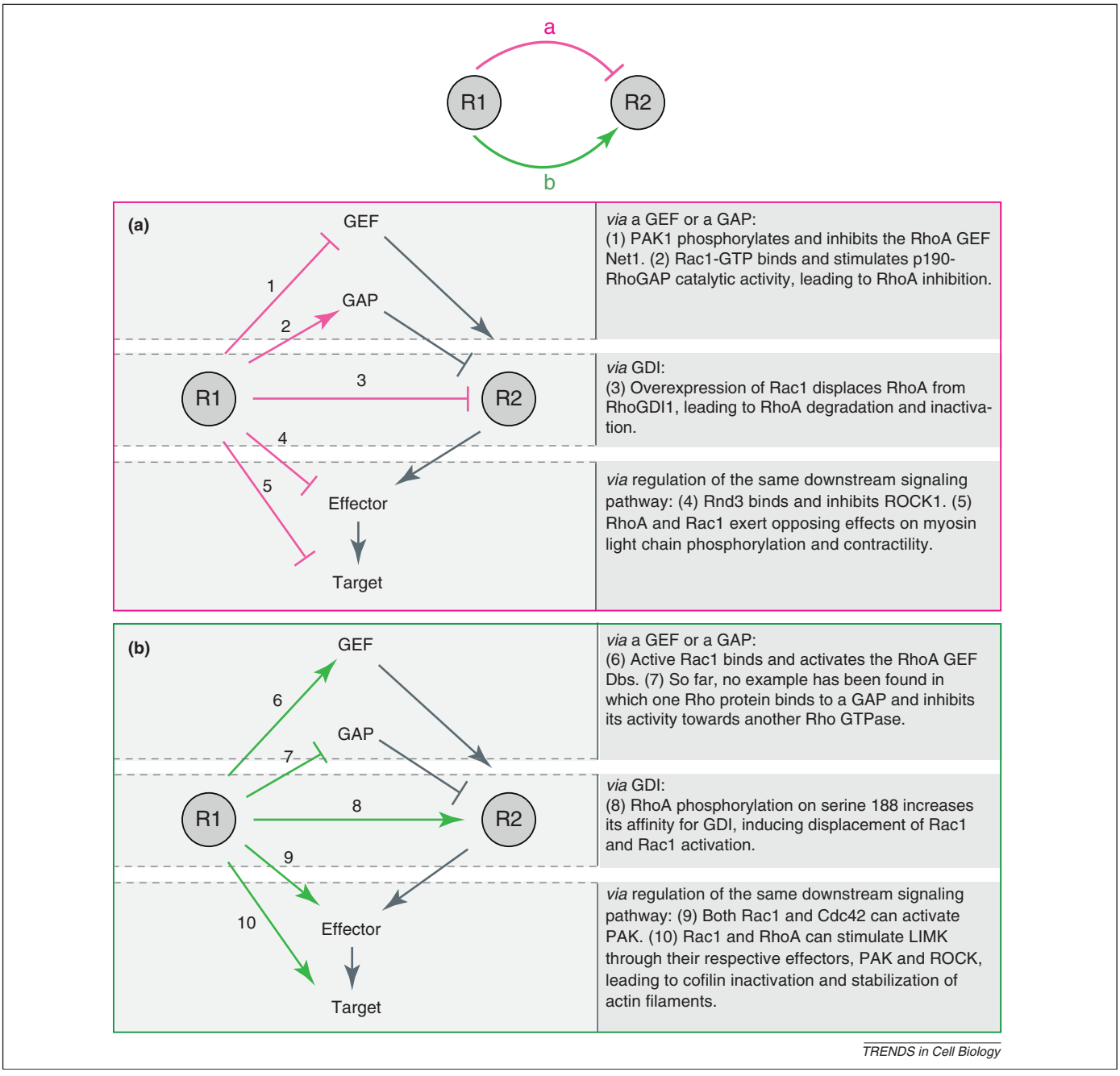

Figure 1. Modalities of regulation between two Rho proteins. Diagram showing how two Rho proteins (R1 and R2) can negatively **(a)** or positively **(b)** regulate one another. An example is indicated for each type of modality (via a GEF or a GAP, via GDI, or via regulation of the same downstream signaling pathway).

due to increased Rac1 activity [8,9]. This led to the idea that ROCK is involved in the suppression of Rac1 activity by RhoA. Pursuing the mechanism for this revealed that ROCK can phosphorylate and activate FilGAP, a Rac-specific GAP [10]. The authors of this work showed that depletion of FilGAP significantly reduced ROCK-dependent Rac1 inactivation. Similarly, a member of the same subfamily of GAPs, ArhGAP22 mediates RhoA-dependent Rac1 inhibition in melanoma cells [11]. ROCK is also responsible for RhoA-dependent ArhGAP22 activation; however, in this case the mechanism may not involve direct phosphorylation of the GAP by ROCK because GAP activation is inhibited by blocking cellular contractility with the myosin II inhibitor blebbistatin, suggesting a more complex mechanism [11].

Another way that mechanical tension can decrease Rac1 activity was suggested by proteomic analysis comparing adhesions under conditions where myosin activity was or was not inhibited. This study showed that the GEF β-Pix was responsible for Rac1 activation in nascent integrin adhesions, and that actomyosin contractility induced β-Pix dissociation from these adhesions [12]. Conversely, ROCK inhibition induced recruitment of β-Pix to the adhesion, indicating that ROCK inhibits Rac1 within the adhesion at least in part by regulating β-Pix localization. In a parallel study, β-Pix and another Rac1 GEF, DOCK180, were seen to be displaced from the large stable adhesions that form at the rear of cells in association with stabilized actomyosin filament bundles [13].

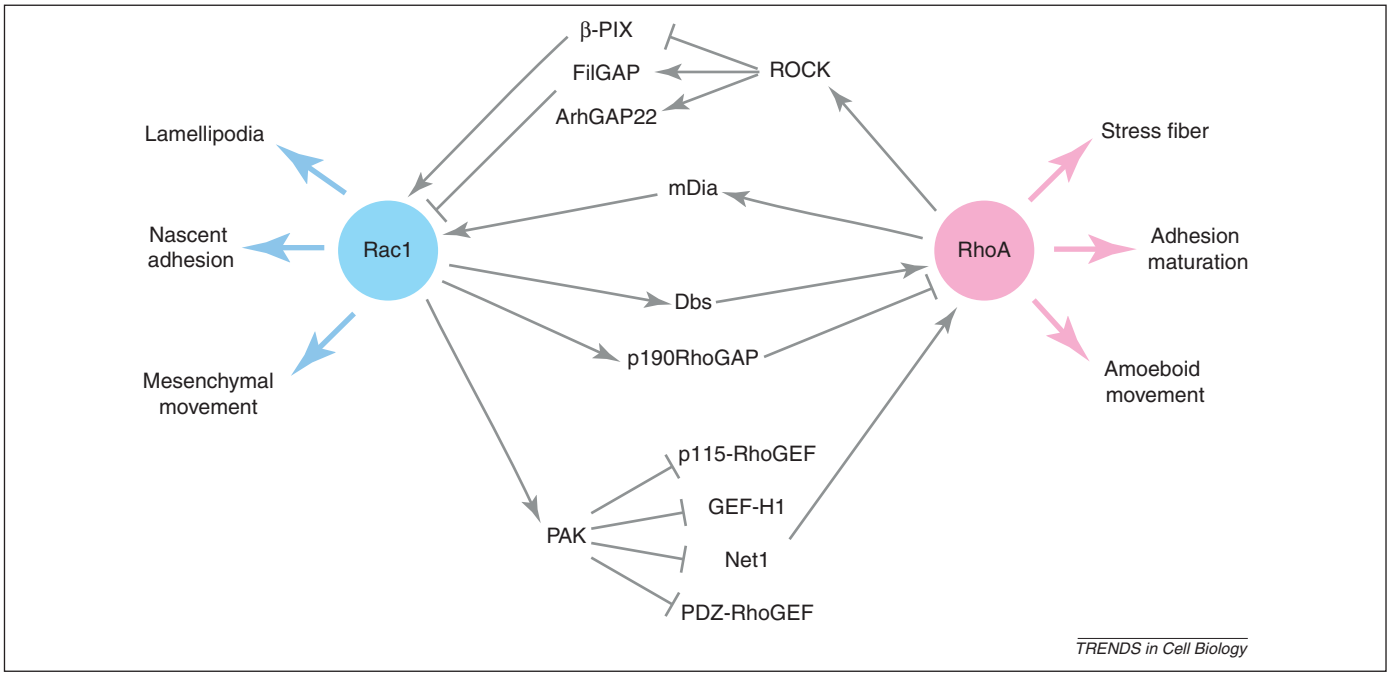

Figure 2. Crosstalk between RhoA and Rac1. Schematic diagram showing the crosstalk mechanisms between RhoA and Rac1.

Rac1 inhibition of RhoA

Mirroring RhoA-dependent Rac inhibition, Rac1 can also control RhoA activity [7]. Active Rac1 binds to and activates p190RhoGAP (isoform B) providing a direct mechanism by which active Rac1 can depress RhoA activity [14]. Interestingly, Rac1 controls p190RhoGAP activity through another mechanism: Rac1-mediated production of reactive oxygen species (ROS) inhibits a tyrosine phosphatase (Low molecular weight protein tyrosine phosphatase), leading to an increase in p190RhoGAP tyrosine phosphorylation and catalytic activity [15]. The effects of ROS on Rho protein activity are complex, because other work showed that direct ROS-mediated oxidation of a cysteine in RhoA leads to RhoA activation rather than inhibition [16]. This latter pathway may occur in situations where there is a positive stimulation of RhoA activity downstream of Rac1.

In the same way as the RhoA effector ROCK mediates some of the downregulation of Rac1 activity, so too the Rac/Cdc42 effector p21 associated kinase (PAK) contributes to Rac1-mediated suppression of RhoA signaling. Notably,

Table 1. Rho GEFs and GAPs whose activity or localization are affected by another RhoGTPase

Name	Type and specificity	Regulated by	Mechanism	Effect
p115-RhoGEF	GEF for RhoA, RhoB and RhoC	Rac1	PAK1 phosphorylates p115-RhoGEF and inhibits p115-RhoGEF-mediated RhoA activation [15]	(–)
GEF-H1	GEF for RhoA, RhoB and RhoC	Rac1	PAK1 and PAK4 phosphorylate GEF-H1 and affect its localization [19,20], leading to RhoA inhibition	(–)
PDZ-RhoGEF	GEF for RhoA, RhoB and RhoC	Rac1	PAK4 phosphorylates PDZ-RhoGEF and inhibits LPA-induced RhoA activation [17]	(–)
Net1	GEF for RhoA	Rac1	PAK1 phosphorylates and inhibits Net1 [16]	(–)
p190-RhoGAP	GAP for RhoA, RhoB and RhoC	Rac1 Rnd1,3	Rac1–GTP binds and activates p190-RhoGAP [12]. Rac1-mediated ROS production stimulates p190-RhoGAP catalytic activity [13] Rnd1 and Rnd3 associate with and activate p190-RhoGAP32	(–) (–) (–)
Dbs	GEF for RhoA and Cdc42	Rac1	Rac1–GTP binds and activates Dbs [21]	(+)
ArhGAP22	GAP for Rac1	RhoA	ROCK-mediated contractility activates ArhGAP22 [10]	(–)
FilGAP	GAP for Rac1	RhoA	ROCK phosphorylates and activates FilGAP [9]	(–)
α-PIX	GEF for Rac1 and Cdc42	Cdc42	Cdc42 activates PAK, which in turn associates with α-PIX, leading to local activation of Rac1 [95]	(+)
β-PIX	GEF for Rac1 and Cdc42	RhoA Cdc42	ROCK-mediated contractility induces β-PIX dissociation from integrin-based adhesion and local Rac1 inhibition [11] Cdc42 promotes PAK association with β-PIX and induces local Rac1 activation [86]	(–) (+)
Dock180	GEF for Rac1	RhoG	ELMO associates with Dock180 and induces its translocation to the plasma membrane, leading to Rac1 activation [25]	(+)

PAK regulates the activities of multiple RhoA-specific GEFs. PAK1 phosphorylates p115-RhoGEF, thereby inhibiting its catalytic activity [17]. Interestingly, this study showed that inhibition of PAK1 significantly increased RhoA activation in response to thrombin, showing that PAK1 may inhibit RhoA activity in physiological contexts; however, this might not exclusively involve p115-RhoGEF downregulation. PAK1 can also phosphorylate the RhoA GEF Net1 on three serine residues [18], and Net1 phosphomimetic mutants have less nucleotide exchange activity toward RhoA *in vitro* and *in vivo*. Another GEF, PDZ-RhoGEF is phosphorylated and inhibited by PAK4 [19], but PAK4 belongs to the group II PAK proteins which have biochemical properties dissimilar to the group I PAKs. PAK4 binds to active Cdc42 and to a lesser extent to active Rac, and binding these GTPases only moderately enhances PAK4 kinase activity [20]. This suggests that *in vivo* PAK4 may inhibit PDZ-RhoGEF independently of Rac1. GEF-H1 (Lfc), another RhoA GEF, is a substrate for both PAK1 and PAK4, and GEF-H1 phosphorylation is associated with decreased RhoA activity, loss of stress fibers, and increased lamellipodia, consistent with increased Rac1 activity [21,22].

Positive feedback between RhoA and Rac1

Although the majority of the mechanisms connecting RhoA and Rac1 lead to mutual inhibition, some studies, including the initial work from Ridley *et al.* [2], have shown that they can also activate each other. Rac1-GTP binds to the PH domain of Dbs, a RhoA GEF [23,24], and stimulates its catalytic activity, leading to RhoA activation. This mechanism seems to be cell type-specific because in breast cancer cells expression of Dbs leads not only to RhoA and Cdc42 activation, but also to Rac1 activation through an unknown indirect mechanism [25]. In contrast to ROCK, mDia (mouse Diaphanous related formin) appears to stimulate Rac1 activity. By comparing the effects of the C3 exoenzyme and the ROCK inhibitor on 3T3 fibroblasts it was found that ROCK inhibition induced Rac1-dependent protrusions, whereas treatment with the C3 exoenzyme did not [9]. Moreover, combined ROCK inhibition and expression of a dominant negative of mDia1 prevented protrusion formation, suggesting that mDia1 positively regulates Rac1 activity. The exact molecular mechanism linking mDia1 to Rac1 is unknown, and does not necessarily involve regulation of a Rac GEF or GAP.

Other Rho proteins that affect Rac1 and RhoA activities

RhoG, which belongs to the Rac subfamily of Rho GTPases, was initially suggested to function by controlling Rac1 activity [26]. ELMO (engulfment and cell motility) was identified as an effector for RhoG [27] and shown to form a complex with Dock180, a Rac-specific GEF [28,29]. *In vivo*, the interaction between Dock180 and ELMO is necessary to stimulate efficient nucleotide-exchange activity on Rac1 [28]. The interaction of RhoG with ELMO induces translocation of the ELMO–Dock180 complex from the cytoplasm to the plasma membrane and activates Rac1 [27]. Thereby, many of the effects of RhoG are due to its downstream activation of Rac1 [30]. However, RhoG can also signal independently or act in parallel, sharing several effectors with Rac1 [31–33].

The Rnd subgroup of Rho proteins, which are only found in vertebrates, are especially interesting in the context of crosstalk because they appear to signal predominantly through the inhibition of RhoA and RhoA-mediated contractility [34]. Unlike other Rho proteins, members of the Rnd subgroup are always bound to GTP and are not controlled by GEFs or GAPs. Instead, the Rnd proteins appear to be regulated at the transcriptional level [34]. In terms of their inhibition of the RhoA signaling pathway, two mechanisms have been identified. One study demonstrated that Rnd1 and Rnd3 bind and activate p190RhoGAP leading to decreased RhoA activity [35]. Rnd3 (RhoE) was independently shown to bind and inhibit the RhoA effector, ROCK1 [36]. In an interesting example of negative feedback, Rnd3 was found to be a substrate for ROCK1 and phosphorylation was shown to enhance Rnd3 stability and promote ROCK1 inhibition [37].

As mentioned above, there are numerous examples where different Rho proteins can be substrates for the same GEF or GAP [3,4]. In addition, some proteins contain multiple domains regulating different GTPases. For example, the Trio family of Rho GEFs contain two GEF domains with distinct specificities, one for RhoA and one for Rac and RhoG [38]. Abr and Bcr both possess a GAP domain, specific for Rac1 and Cdc42, and a GEF domain, specific for RhoA, Rac1 and Cdc42 [39]. Recently, it was shown that Abr regulates local RhoA activation and Cdc42 inactivation during the wound-healing response in *Xenopus* oocytes [40]. During the closure of a small wound in an oocyte, a zone of active RhoA surrounds the wound that is, in turn, encircled by a region of active Cdc42. Abr is responsible for the zone of active RhoA and for inactivating Cdc42 within this region [40]. With regulators that have multiple catalytic domains, it would be interesting to know if the activity of one influences the activity of the other. For example, does Cdc42 binding to the GAP domain of Abr or Bcr affect the activity of the GEF domain specific for RhoA?

Crosstalk via GDI

Compared to GEFs and GAPs, the family of RhoGDI proteins acts very differently on Rho GTPases [6] (Figure 1). Whereas the number of GEFs and GAPs greatly outnumber the GTPases, there are only three conventional GDIs, immediately implying that many GTPases can bind to a single GDI. RhoGDI1 (RhoGDIα) is ubiquitous and is the most studied. It appears to bind most Rho GTPases, although its interaction with RhoB differs in different studies and may reflect that RhoB can be palmitoylated close to its C terminus and this palmitoylation would probably block binding to RhoGDI [41]. RhoGDI2 (RhoGDIβ, Ly-GDI, D4-GDI) is predominantly found in hematopoietic tissues, but is also expressed in numerous tumors. RhoGDI3 (RhoGDIγ) is expressed at low levels in many tissues but particularly in the brain, lungs and testes. RhoGDI1 and RhoGDI2 are cytosolic proteins, but RhoGDI3 contains an N-terminal sequence extension that associates it with intracellular membranes such as the Golgi and endosomes. It appears to interact predominantly with RhoB and RhoG. As their name implies, RhoGDIs

function to inhibit nucleotide dissociation from Rho GTPases. They prevent GEF-mediated exchange and inhibit GAP activity. For RhoGDI1 and 2, a crucial function is their ability to extract Rho GTPases from membranes, where normally the GTPases interact with downstream effectors. Recent studies have revealed that RhoGDI1 promotes the stability of Rho GTPases and protects them from degradation [42,43]. Interestingly, RhoGDI1 is expressed in cells at a level that is approximately equal to the sum of the major Rho family members [41]. This implies that it acts as a limited reservoir for the Rho proteins, with individual members competing for binding. Overexpression of one Rho family member was found to displace other Rho proteins from RhoGDI1, leading to their degradation and inactivation [43]. In some situations, however, the competitive displacement of one Rho protein by increased binding of another activates the displaced GTPase [44]. Phosphorylation of RhoA on serine 188 by PKA or PKG increases the affinity of RhoA binding to RhoGDI1, and in vascular smooth muscle this was shown to displace bound Rac1, which was then translocated to the membrane and activated by the GEF, Vav3 [44]. This result suggests that the competition for binding to RhoGDI by Rho proteins may not be as coarse a regulatory mechanism as was originally envisaged, but may allow modifications in binding affinity of one Rho protein to modulate the release and stability and/or activation of others.

The competitive binding to RhoGDI provides a mechanism for crosstalk between Rho proteins at the level of protein stability and degradation. The literature suggests that crosstalk may also occur through as yet uncharacterized transcriptional pathways. There is evidence that this may occur within the Rho subfamily, which includes RhoA, B and C [42,45]. These closely related proteins share particular characteristics, such as the ability to induce stress fibers, but they also have unique functions. RhoA, for example, is required for mitosis in fibroblasts and cannot be substituted by RhoB or RhoC [46]. In some situations, these family members can exhibit opposite functions, as illustrated by the effects of RhoA and RhoC on cell migration and invasion [45]. Similarly, RhoB has the properties of a tumor suppressor, being pro-apoptotic, whereas RhoA and C have characteristics closer to being oncogenes [47]. Depletion of either RhoA or RhoC expression leads to a marked increase in RhoB levels. With the individual depletion of either RhoA or RhoC, little effect on the transcription of RhoB was observed, but simultaneous depletion of both proteins greatly increased RhoB mRNA levels [42], indicating significant regulation at the transcriptional level that will be interesting to explore further.

A study on the mechanism by which RhoB expression is influenced by RhoA or RhoC levels discovered that the half-life of RhoB, which is normally short-lived, is greatly increased by the knockdown of these other family members. RhoGDI1 is the crucial component mediating the interaction between these family members [42]. Overexpression of RhoGDI1 increased the half-life of RhoB, whereas its depletion inhibited the effect of RhoA on RhoB expression, leading to the conclusion that RhoGDI stabilizes RhoB against degradation [42]. Although this study provided evidence that the crosstalk did not involve RhoA activation [42], a different group observed that inhibiting Rho activity with the C3 toxin also increased RhoB expression, implying that active Rho proteins are required for some part of this interaction [45].

Crosstalk via regulation of the same downstream signaling (target or effector)

In this type of crosstalk, two (or more) Rho proteins share the same effector or molecular target (Figure 1).

Regulation of the same effector

Rac and Cdc42 have in common many effectors, including PAK1-3, Ncf1/2 and IQGAP [5]. RhoA and Rac have also been shown to share some downstream effectors. Early work on the kinase PRK2 showed that it interacts with both Rac and RhoA, in both cases leading to stimulation of its kinase activity [48]. More recent studies, however, observed that PRK2 predominantly acts downstream of RhoA *in vivo* to regulate apical junctions [49], suggesting that the crosstalk occurring through PRK2 may only take place under specific circumstances *in vivo*. The mDia formins are regulated by multiple Rho proteins, and RhoA, B, and C [50] and Rif [51] can activate mDia1, whereas, in addition to RhoA, Rif [52], Rac and Cdc42 [50] activate mDia2. Surprisingly, this occurs through interaction with the same domain [50], despite their weak similarity.

Regulation of myosin light chain (MLC) phosphorylation

In nonmuscle cells, myosin II-generated tension is regulated by MLC phosphorylation. Several kinases have been identified that promote this phosphorylation, either directly or indirectly by inhibiting the MLC phosphatase. These include ROCK1 and 2, which are activated by RhoA, and MRCK, which is activated by Cdc42 [53]. The Rac and Cdc42 effector PAK, on the other hand, can have opposite effects on MLC phosphorylation depending on the cell type analyzed. In Hela and BHK cells, PAK inhibits MLC phosphorylation and cell contractility by phosphorylating and inhibiting the MLC kinase [54], whereas in 3T3 and endothelial cells PAK increases MLC phosphorylation [55,56], although the mechanism has not been determined. More recently, it was shown that Rac1 inhibits MLC phosphorylation in melanoma cells, however, in this study WAVE2 mediated this effect rather than PAK1, 2 or 3 [11]. Together, these results suggest that Rac and RhoA may act synergistically on MLC phosphorylation and cellular contractility in some cells but antagonistically in others.

Regulation of cofilin

The ADF/cofilin family of actin binding proteins promote actin filament disassembly [57]. In 1998, two groups independently reported that Rac1 regulates actin dynamics through, in part, phosphorylation mediated by the LIM (Lin11, Isl-1 and Mec-3) kinase LIMK1 and inhibition of cofilin. Working *in vivo* and *in vitro*, both groups showed that Rac1 activates LIMK1, which in turn phosphorylates cofilin [58,59] on serine 3 and decreases its binding to actin. It was subsequently shown that both Rac1 and Cdc42 regulate LIMK activity through PAK1 [60] and that RhoA can also regulate LIMK2 [61] and LIMK1 [62] via ROCK-dependent phosphorylation, leading to phosphorylation

and functional inhibition of cofilin. These findings demonstrate that RhoA, Rac1 and Cdc42 act synergistically on cofilin and control actin filament stability through LIMK-dependent phosphorylation of cofilin. This crosstalk between RhoA, Rac and Cdc42 plays a central role during neuronal growth [57].

Rho protein coordination in cell adhesion and motility

Integrin-based adhesion: switching from Rac1 to RhoA

Since the discovery that Rho proteins control cell adhesion to the extracellular matrix (ECM) [63,64], the relationship between individual Rho proteins and the assembly of adhesions has been extensively investigated. As cells adhere to the ECM, RhoA and Rac play distinct and opposing roles. Rac promotes formation of nascent adhesions and couples these small adhesions (<0.5 μm) with actin-based protrusion near the cell periphery, whereas RhoA-dependent contractility produces changes in adhesion composition, leading to formation of larger (>1 μm) more mature focal adhesions [65,66] (Figure 3). Consistent with these respective roles, initial adhesion and spreading are associated with transient RhoA inhibition [67] and Rac1 activation [68], followed later by gradual RhoA activation and Rac1 inhibition. The balance between RhoA and Rac1 appears to control the fate of adhesions, and much effort has been directed recently to identifying the GEFs and GAPs that orchestrate this switch.

Different pathways have been proposed for the regulation of Rac1 during the early phase of adhesion and various GEFs and GAPs have been suggested to play a role, including β-PIX [12,69], α-PIX [70] and CdGAP [71]. Since the identification of the DOCK180/ELMO pathway [27], RhoG has been suggested to activate Rac downstream of integrin engagement [72]. It was recently shown, however, that RhoG depletion does not affect adhesion-dependent Rac activation [73]. Concomitant with Rac activation, we and others showed that RhoA inhibition is mediated by p190RhoGAP [74–76]. Interestingly, Rac regulates p190RhoGAP activity and localization directly [14], and also indirectly through ROS generation [15]. This suggests that Rac may contribute to inhibit RhoA locally by activating and recruiting p190RhoGAP to nascent adhesions. More recently it was found that PKA is activated in an adhesion-dependent manner at the leading edge of migrating epithelial cells and phosphorylates RhoA on Ser 188, increasing its affinity for GDI [77]. The authors showed that the resulting increased association of RhoA with GDI inhibits RhoA. As a consequence of the competitive binding on GDI that occurs between Rho proteins (see discussion above), one could anticipate that PKA-dependent RhoA phosphorylation might also induce Rac1 dissociation from GDI [43] and its activation [78]. Consistent with this idea, integrin-dependent adhesion was discovered to promote Rac1 dissociation from GDI, leading to interaction with its effectors [79].

Subsequent to this first phase controlled by Rac1, Rac1 activity decreases whereas RhoA becomes activated, promoting the growth and maturation of the nascent adhesions

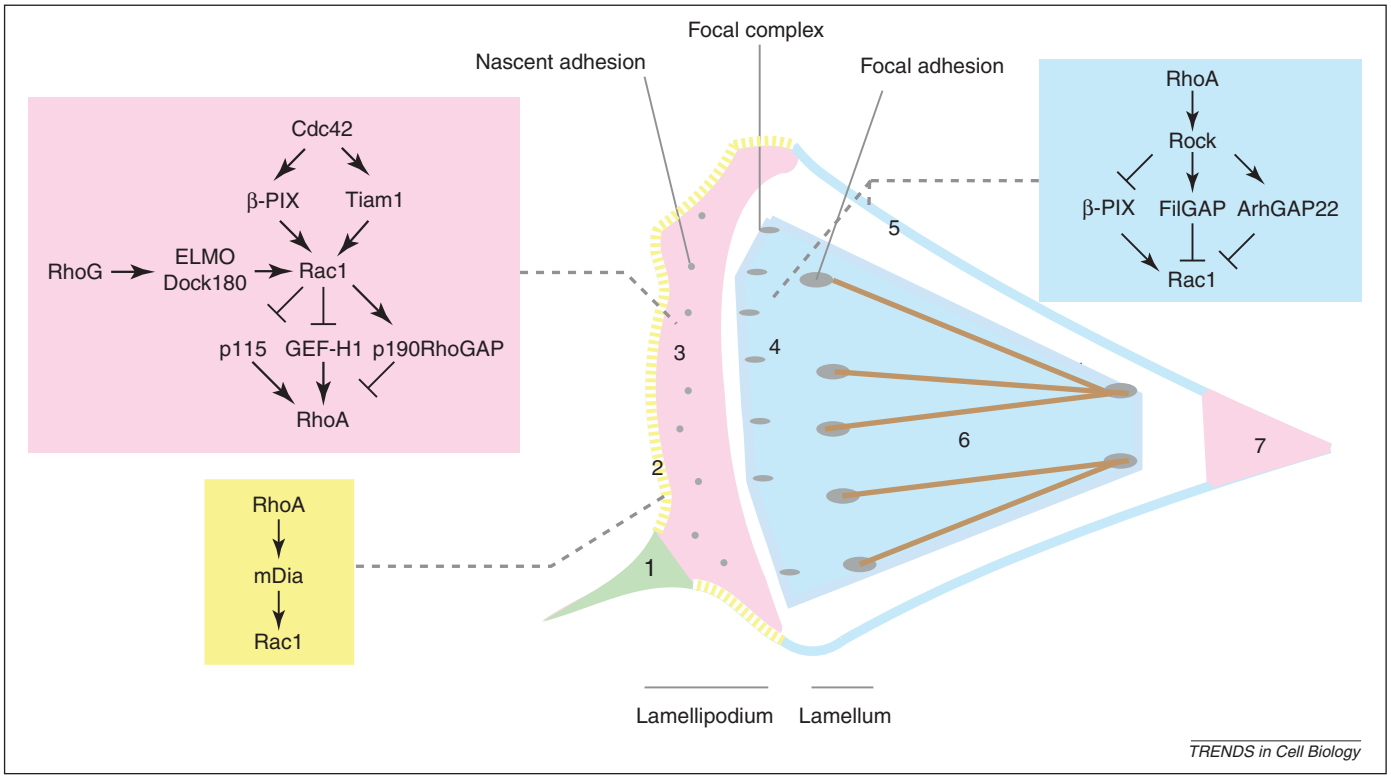

Figure 3. Rho protein crosstalk during cell migration. A diagram of a migrating fibroblast is shown depicting zones of Rho protein activation and crosstalk. **(1)** Cdc42 controls formation of exploratory filopodia. **(2)** RhoA activity has been detected at the leading edge of lamellipodia where it may contribute to actin polymerization, directly *via* mDia or indirectly through mDia activating Rac1. **(3)** Behind the narrow zone of high RhoA activity, a wider zone of high Rac1 activity has been described. This may arise downstream from integrin engagement. Alternatively, Cdc42 and RhoG may contribute to Rac1 activation. This, in turn, inhibits RhoA and promotes nascent adhesion formation associated with actin-based protrusion. **(4)** RhoA generates ROCK-mediated contractility and inhibits Rac1, leading to adhesion maturation. **(5)** RhoA prevents inappropriate lateral protrusion by inhibiting Rac1 through ROCK2. **(6)** RhoA promotes cell body retraction through ROCK1-mediated myosin II stimulation. **(7)** Rac1 activation at the tail has been described but its function in this area is unknown.

into focal adhesions. Different GEFs have been suggested to play a role in this process including LARG [80], p115RhoGEF [80], p190RhoGEF [75] and GEF-H1 [12]. Interestingly, the Rac/Cdc42 effector PAK regulates the activity of p115RhoGEF [17] and GEF-H1 [21,22], suggesting that Rac could potentially prevent RhoA activation and adhesion maturation during early spreading by inhibiting these two GEFs. Recent evidence suggests that, as adhesion progresses, RhoA activation may control the local inhibition of Rac. A mass spectrometry approach revealed how the adhesion proteome changes upon myosin inhibition [12]. It was found that β-PIX recruitment to adhesions is negatively regulated by contractility. These results suggest that RhoA-dependent myosin II activation triggers β-PIX dissociation from the adhesion and inhibits Rac1 locally [12]. Certainly, the development of mechanical tension contributes in several ways to the maturation of adhesions. Recent work has demonstrated that tension applied to integrins activates both GEF-H1 and LARG, although via different pathways, contributing to increased RhoA activity [81].

Cell migration

Cell migration can be divided into distinct steps: protrusion of the leading edge, formation of new adhesions, cell body contraction and rear detachment [82]. Because actin cytoskeleton dynamics constitute the driving force during these steps, it is not surprising that Rho proteins have been implicated in regulating cell migration. Numerous studies have demonstrated that the prototypical members of the Rho family, RhoA, Rac1 and Cdc42, have specific roles during cell migration [1]. Recently, the development of fluorescent resonance energy transfer (FRET)-based biosensors that allow the visualization of spatiotemporal Rho signaling has demonstrated that RhoA, Rac1 and Cdc42 signal within distinct and specific zones during cell migration [83–86]. This confirms and spectacularly illustrates the idea that Rho proteins cooperate during migration [87], suggesting that coordination and crosstalk between the Rho family members are essential to achieve efficient movement (Figure 3).

Rac1 regulates actin polymerization in the lamellipodial protrusion and promotes the formation of nascent adhesion near the cell periphery [88]. Using live-cell imaging of Rac biosensors in migrating neutrophils it was found, as expected, that Rac1 is active in the extending leading edge [85] of the cell. However, the authors also observed Rac1 activity at the rear where there is no actin-based protrusion [85], indicating that Rac1 may play a different role in these two areas. Nevertheless, photoactivation of a caged constitutively active Rac1 construct in any region of a fibroblast appears sufficient to induce lamellipodial extension [89].

Cdc42 has been shown to regulate the polarity of cell migration through different mechanisms. Cdc42 was shown to limit Rac1 activity at the front of migrating cells through PAK-mediated regulation of the Rac-specific GEF β-Pix [90]. The same group also showed that Cdc42 regulates microtubule polarity during directed migration by activating the atypical PKC in the Par6/aPKC complex at the leading edge [91,92]. Interestingly, the Rac GEF Tiam1 associates with the Par complex and is necessary for polarity establishment during keratinocyte migration [93], suggesting that Cdc42 may control cellular polarity primarily through defining Rac1 activation area.

RhoA appears to play a role in every cellular compartment during cell migration [1]. RhoA is most commonly considered in the context of generating the contractile force that promotes cell body retraction at the rear through ROCK-mediated MLC phosphorylation [94]. However, it can also contribute to the extension of the leading lamella, as shown first with colon carcinoma cells migrating on laminin [95]. FRET-based biosensor imaging revealed that RhoA is active at the leading edge [83]. Through its interaction with the formin, mDia, RhoA drives actin polymerization [96], but mDia has also been shown to activate Rac1 [9], providing another example of RhoA crosstalk with Rac1 in the coordination of migration. RhoA also contributes to maintain cellular polarity by limiting inappropriate lateral protrusion [8,45]. Most likely this occurs through ROCK-mediated Rac1 inhibition via local inhibition of the Rac GEF β-PIX [12,13] and/or activation of the Rac GAPs Arhgap22 [11] or FilGAP [10]. Recently, it was shown that the different ROCK subtypes mediate different functions, with a role for ROCK1 in promoting cell body retraction, and ROCK2 inhibiting Rac1 and preventing protrusion [45]. For protrusion to occur at the front of cells it is presumably important that the inhibitory function of ROCK2 is suppressed. Consistent with this idea, ROCK2 is inhibited by adhesion-induced tyrosine phosphorylation [97], which can be anticipated to occur as the front of an advancing cell engages the ECM. Thus, RhoA plays distinct roles at the front and at the back of migrating cells, most probably through interacting with different sets of effectors at the different sites.

Working with melanoma cells, new crosstalk mechanisms were identified between RhoA and Rac1 [11]. Depending on the environmental conditions, individual tumor cells have two modalities of movement: a mesenchymal mode characterized by an elongated morphology, and an amoeboid mode associated with high ROCK activity [98]. The authors showed that the mesenchymal mode is controlled by Rac1, which signals to WAVE2 to inhibit contractility and the amoeboid mode of migration [11]. Conversely, during amoeboid movement, ROCK inhibits Rac1 by stimulating the Rac GAP, ArhGAP22.

Concluding remarks

Although a considerable amount has been learned about the crosstalk between different Rho GTPases, much of this understanding is at the upstream level involving GEFs and GAPs. By contrast, less is known about the crosstalk that occurs downstream and that involves the interactions of the various signaling pathways initiated by the Rho GTPases. It is anticipated that there is much to be uncovered about crosstalk at the level of Rho protein effectors. In addition, we expect that there is more to learn about how Rho GTPases can affect each other's expression, either at the transcriptional level or by influencing protein stability and degradation. When considering a complex behavior such as cell migration it is striking that many levels of crosstalk often take place simultaneously in different regions of the cell, such as at the leading edge, within the different types of

adhesions, at the cell margins, and in the cell rear. Many of these sites of crosstalk involve distinct protein complexes. The role of scaffold proteins in the assembly of these complexes and how they may contribute to the regulation of Rho protein crosstalk is poorly understood but promises to be a rich area of future investigation.

The crosstalk between Rho family members described in this brief review represents just a small corner of the interactions between members of the Ras superfamily. Indeed, the first paper describing an interaction between Rac and Rho also revealed that Ras itself can signal to Rac [2]. Numerous additional relationships have been discovered in which Ras family GTPases, including Rab, Arf and Rap GTPases, affect the activities and signaling of Rho GTPases and vice versa. This interdependence helps to coordinate a vast array of cellular processes, including migration, adhesion, membrane traffic and cell division. The proliferation of crosstalk mechanisms within the Ras superfamily parallels the expansion of metazoans and has been accompanied by the emergence of new family members. Our understanding of this network of interactions will continue to increase, being driven by technological advances in proteomics, live-cell imaging and systems biology.

Acknowledgments

The authors apologize to the many groups whose work they could not include due to space limitations. C.G. thanks the European Commission Seventh Framework Programme (FP7/2007–2013) for a Marie Curie Outgoing International Fellowship (254747). R.G.M. gratefully acknowledges support from the Simmons Scholars Program; K.B. thanks the Kenan Foundation for support and support from grants from the National Institutes of Health (GM029860 and HL080166).

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