Mammalian Rho GTPases: new insights into their functions from *in vivo* studies

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Abstract | Rho GTPases are key regulators of cytoskeletal dynamics and affect many cellular processes, including cell polarity, migration, vesicle trafficking and cytokinesis. These proteins are conserved from plants and yeast to mammals, and function by interacting with and stimulating various downstream targets, including actin nucleators, protein kinases and phospholipases. The roles of Rho GTPases have been extensively studied in different mammalian cell types using mainly dominant negative and constitutively active mutants. The recent availability of knockout mice for several members of the Rho family reveals new information about their roles in signalling to the cytoskeleton and in development.

Mammalian Rho GTPases comprise a family of 20 intracellular signalling molecules, best documented for their important roles in regulating the actin cytoskeleton. Most Rho GTPases switch between an active GTP-bound form and an inactive GDP-bound form. The cycling of Rho GTPases between these two states is regulated by three sets of proteins, guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs) (BOX 1). Rho proteins interact with and activate downstream effector proteins when bound to GTP, thereby stimulating a variety of processes, including morphogenesis, migration, neuronal development, cell division and adhesion. In addition, they regulate vesicle transport, microtubule dynamics, cell-cycle progression and gene expression[1].

Although most studies have focused on the so-called 'classically activated' Rho GTPases, eight members of the family are described as 'atypical' (FIG. 1). These atypical proteins are predominantly GTP-bound owing either to amino-acid substitutions at residues that are crucial for GTPase activity (for example, in Rnd proteins and RhoH) or owing to increased nucleotide exchange (for example, in WNT1-responsive CDC42 homologue-1 (WRCH1), also known as RhoU[2]). Therefore, these proteins are not thought to be regulated by GEFs and GAPs. Instead, gene expression, protein stability and phosphorylation regulate these Rho GTPases[3].

Much of our understanding of the roles of mammalian Rho GTPases has come from overexpression studies in cell lines with dominant negative and constitutively active Rho GTPases, which inhibit or overstimulate Rho GTPase signalling, respectively (BOX 1). Loss-of-function mutants in model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have provided insights into the *in vivo* functions of the most highly conserved Rho GTPases — RhoA, Rac and CDC42 — but not into other family members, most of which are absent from these organisms[4]. The generation of knockout mice for several Rho GTPases has provided new tools to study the function of single isoforms of often highly homologous proteins (FIG. 1, Supplementary information S1 (table)), as well as allowing the *in vivo* analysis of these proteins. The recent development of CDC42 and RAC1 conditional knockouts has been particularly important, because full knockout of these highly conserved proteins is lethal early in embryogenesis. We discuss new insights into the functions of Rho GTPases gained from analysis of knockout phenotypes, and compare these findings with the previously described roles of Rho GTPases that were based on studies with dominant negative and constitutively active mutants.

CDC42

CDC42 has a conserved role in regulating cell polarity and the actin cytoskeleton in many eukaryotic organisms. Cdc42 has been shown to have a role in yeast budding, epithelial polarity, migratory polarity and fate specification during cell division. Cdc42 was first identified in *Saccharomyces cerevisiae* as a cell-cycle mutant; loss of Cdc42 prevents budding and mating

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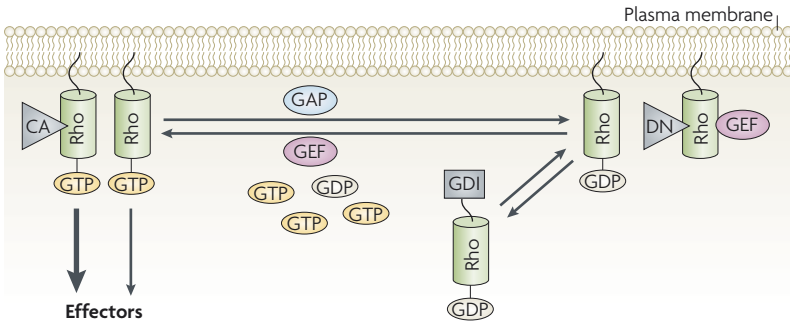
doi:10.1038/nrm2476

Box 1 | **Regulation of Rho GTPases**



Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the release of GDP and the binding of GTP[144]. Over 70 GEFs have been described in humans, most of which contain a Dbl-homology (DH) domain and an adjacent pleckstrin homology (PH) domain, which catalyse GDP–GTP exchange. A second group of GEFs is the 180 kDa protein downstream of CRK (DOCK180)-related proteins that contain two Dock-homology regions (DHR)[145]. Many GEFs can activate multiple Rho GTPases — for example, T-cell-lymphoma invasion and metastasis-1 (TIAM1) activates RAC1, RAC2 and RAC3, and VAV1 activates RhoA, RAC1, RhoG and CDC42 (REF 144). Dominant-negative (DN) Rho GTPases contain a substitution mutation of Thr for Asn at amino acid 17 (RAC1 numbering). This mutation allows binding to GEFs but inhibits downstream interactions with effector proteins, so that a dominant-negative Rho GTPase titrates out the GEFs that bind to that Rho GTPase[146]. Because GEFs can function on several Rho GTPases and in turn Rho GTPases can be activated by multiple GEFs[144], overexpression of a dominant-negative protein could prevent GEFs from activating other Rho GTPases.

GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity of Rho proteins. There are over 80 mammalian GAPs, of which some function preferentially on one GTPase (such as CDC42GAP), whereas others are more promiscuous[147]. Constitutively active (CA) mutants cannot hydrolyse GTP and therefore signal constitutively to their effector proteins. Common constitutively active mutations are Gly to Val at amino acid 12 (RAC1 numbering) or Gln to Leu at amino acid 61. Overexpression of constitutively active mutants could sequester effector proteins and inhibit signalling by other Rho GTPases that share these effectors[148].

Guanine nucleotide-dissociation inhibitors (GDIs) bind to C-terminal prenyl groups on some Rho proteins, sequestering them in the cytoplasm away from their regulators and targets. There are three RhoGDIs in mammals[149].

projection[5]. Although CDC42 is not present in two important models that are used to study cell polarity — *Arabidopsis thaliana* and *Dictyostelium discoideum*[4] — it is essential for early development in *D. melanogaster* and *C. elegans*[6,7]. *Cdc42*-knockout mice are embryonic lethal and die before embryonic day 7.5 (REF. 8), and thus tissue-specific *Cdc42*-knockout models have been made to study CDC42 function beyond early embryogenesis (Supplementary information S1 (table)).

Regulating filopodium formation. In many cell types, both constitutively active CDC42 and dominant-negative CDC42 affect the formation of highly dynamic finger-like actin-rich protrusions known as filopodia[9]. Other Rho GTPases also induce filopodium formation, including RhoQ, RhoU, RhoF and RhoD[10–12]. Filopodia contain parallel bundles of filamentous (F)-actin (FIG. 2a), and are thought to be important for sensing the environment, for example, in axon guidance or the formation of epithelial cell–cell contacts[9].

For the most part, the results of studies on filopodia in CDC42-null cells concur with results obtained from dominant-negative CDC42 studies. For example, CDC42-null embryonic stem cells (ESCs) show defects in the organization of the actin cytoskeleton, including a reduction in the number and size of filopodium-like protrusions[8]. CDC42-null neurons have reduced filopodia[13]. CDC42-null mouse embryonic fibroblasts (MEFs) completely lack filopodia, whereas MEFs from a CDC42GAP-null mouse with increased CDC42 activity have high levels of spontaneous filopodium formation[14]. By contrast, CDC42-null ESC-derived fibroblastoid cells form normal filopodia and lamellipodia[15]. Dominant-negative CDC42 also failed to prevent filopodium formation in fibroblastoid cells, which suggests that a CDC42-independent mechanism, perhaps involving RhoF, could be used to form filopodia in these cells[9].

Several downstream targets for CDC42 have been implicated in filopodium formation (FIG. 2b). Wiskott–Aldrich syndrome protein (WASP), which activates the actin-related protein-2/3 (ARP2/3) complex, was initially thought to be the main contender, but cells that lack both WASP isoforms protrude filopodia normally[16]. Subsequently, mammalian diaphanous (mDia) proteins — members of the formin family — were found to stimulate the polymerization of unbranched actin filaments, and the CDC42 target mDia2 was shown to mediate filopodium formation through studies on mDia1-null cells[17]. The protein IRSp53 (insulin-receptor substrate p53) is a CDC42 target that also contributes to filopodium formation, both by bundling actin filaments and by inducing membrane curvature[18].

Neurite extension, axon growth and filopodia. One predicted physiological role for filopodia is to function as sensory probes during the directed migration of neuronal growth cones[19]. Studies of dominant negative and constitutively active mutants have implicated CDC42 in filopodium extension in growth cones and in multiple aspects of neuronal development, including neurite extension, axon specification and axon guidance[20] (FIG. 3). Widespread deletion of CDC42 in the brain using a conditional knockout is lethal at birth and results in a large decrease of axon numbers and in a reduced size of the brain cortex[13]. Analysis of cultured hippocampal neurons from these knockout mice revealed that CDC42 is required for axon generation; however, dendrites and minor neurites seem to have been unaffected.

CDC42-null neurons have few or no filopodia and increased cofilin phosphorylation. Cofilin regulates actin dynamics by stimulating actin-filament severing and depolymerization, and is inactivated by phosphorylation. Cofilin is known to be required for filopodial dynamics in growth cones[21,22], and overexpression of wild-type cofilin or cofilin that cannot be phosphorylated increased axon growth[13], which suggests that CDC42 could stimulate axonal growth by reducing cofilin phosphorylation, which in turn increases filopodial extension. However, this model is in contrast to the well-characterized pathway in which CDC42 activates LIM kinase to stimulate cofilin phosphorylation and inactivation[23] (FIG. 2b) and hence reduces axon

Formins
A family of proteins that bind to the barbed end of actin filaments and regulate actin dynamics.

Actin-filament severing
The disruption of interactions between neighbouring actin molecules in actin filaments such that the filament is cut in two. Actin-filament severing proteins include gelsolin and villin.

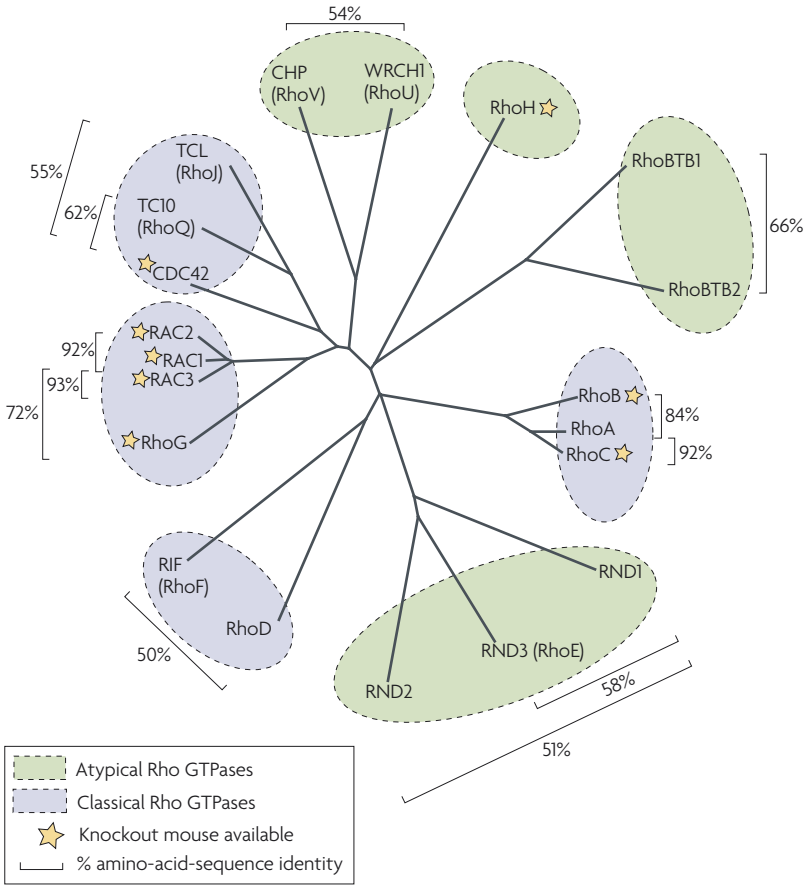


Figure 1 | **Rho GTPase family.** An unrooted phylogentic tree that is based on the ClustlW alignment of the amino-acid sequences of the 20 Rho GTPase proteins. The tree demonstrates the relationship between the different family members. The Rho GTPases form 8 subfamilies; subfamily one comprises RAC1, RAC2, RAC3 and RhoG; subfamily two comprises CDC42, TC10 (also known as RhoQ) and TC10-like protein (TCL; also known as RhoJ); subfamily three comprises CHP (also known as RhoV) and WNT1-responsive CDC42 homologue-1 (WRCH1; also known as RhoU); subfamily four comprises RhoH; subfamily five comprises RhoBTB1 and RhoBTB2; subfamily six comprises RhoA, RhoB and RhoC; subfamily seven comprises RND1, RND2 and RND3 (also known as RhoE); and subfamily eight comprises RAP1-interacting factor-1 (RIF; also known as RhoF) and RhoD. EMBOSS pairwise alignment was used to calculate the percentage of amino-acid-sequence identity within subfamilies. High sequence similarity is found between proteins within the Rac and Rho subfamilies, whereas the other subfamilies are much less similar. The classical Rho GTPases include the Rho, Rac, CDC42 and RhoF and RhoD subfamilies and these all cycle between active GTP-bound forms and inactive GDP-bound forms. The atypical Rho GTPases comprise the RhoBTB, Rnd, RhoU and RhoV subfamilies and RhoH. These proteins are all effectively GTP-bound and are thought to be regulated by other mechanisms, including phosphorylation and protein levels.

growth[24]. How the loss of CDC42 in mouse neurons leads to increased cofilin phosphorylation remains unknown. It is unlikely that CDC42 affects cofilin through changes in RAC1 or Rho signalling, as RAC1 activity is not affected in CDC42-null neurons and the addition of Rho inhibitors did not rescue their phenotype[13]. It is possible that it occurs through an indirect upregulation of another Rho GTPase, such as RhoQ or RhoJ (FIG. 1), or deregulation of a cofilin phosphatase. Together, these data point to a new link between CDC42 and cofilin in axon extension.

Glial cells
Non-neuronal cells of the nervous system that provide support and nutrition for neurons. They also form myelin and contribute to axon guidance.

Axon myelination. A novel function for CDC42 has been suggested by studies of glial cells, which wrap multiple layers of their plasma membrane around axons to form myelin[25]. In oligodendrocytes — the glial cells of the central nervous system — myelin sheaths are thinner in the absence of CDC42, which seems to be the result of a defect in removing cytoplasm as the oligodendrocyte plasma membranes wrap around the axon[26]. Although the mechanistic basis for this phenotype is unknown, it is possible that CDC42-driven cortical actomyosin contraction could push cytoplasm out as the membranes extend around the axon. Schwann cells — the glial cells of the peripheral nervous system — that lack CDC42 are also defective in axon myelination. However, CDC42 is required for Schwann-cell proliferation, which precludes analysis of the role of CDC42 during myelination[27].

Migration and chemotaxis. CDC42 has been implicated in chemotaxis and directed migration of several cell types both *in vitro* and *in vivo*, including macrophages, T cells, fibroblasts and *D. melanogaster* haemocytes[28–32]. Studies on CDC42-deficient MEFs and haematopoietic stem cells (HSCs) support a central role for CDC42 in chemotaxis[14,33]. By contrast, directed migration of CDC42-null fibroblastoid cells was reported to be normal[15], which indicates that the contribution of CDC42 could be cell-type-specific. CDC42 could affect chemotaxis by decreasing Rac localization and activation to the leading edge, thereby reducing the stability of lamellipodia so that cells cannot move persistently in one direction[32,34]. Indeed, a common observation in CDC42-null cells is a reduction of RAC1 activity[15,35]. Conversely, an increase in CDC42 activity can stimulate migration speed, for example in neutrophils isolated from *Cdc42GAP*-knockout mice[36] and RAC2-null HSCs/progenitor cells (HSC/P cells)[37]. This could be due to the ability of overactivated CDC42 to stimulate Rac-dependent lamellipodial protrusion.

CDC42-null or CDC42GAP-null leukocytes show alterations in recruitment to inflammatory sites and/or homing *in vivo*[33,36], but it is unknown whether these defects reflect changes to adhesion, migration or chemotaxis. Loss of CDC42 strongly affects haematopoietic cell differentiation and cell-cycle progression[33], both of which could indirectly affect the migratory capacity of cells.

Regulating polarity through the PAR complex. Cell polarization is the process by which a cell responds to an extracellular stimulus by redistributing and maintaining proteins and organelles in an asymmetrical layout. Polarity is fundamental to many cellular processes, including migration, differentiation and morphogenesis. CDC42 seems to function primarily through the polarity protein partitioning-defective-6 (PAR6) and thereby with PAR3 and/or atypical protein kinase C (aPKC) isoforms to induce polarity in several different animal models[5,38]. For example, CDC42 and the PAR complex (PAR6–PAR3–aPKC) have been proposed to mediate the capture and stabilization of microtubules at the front of the cell and to orientate the Golgi and microtubule-organizing centre (MTOC) during the establishment of migratory polarity[39,40]. CDC42 can also

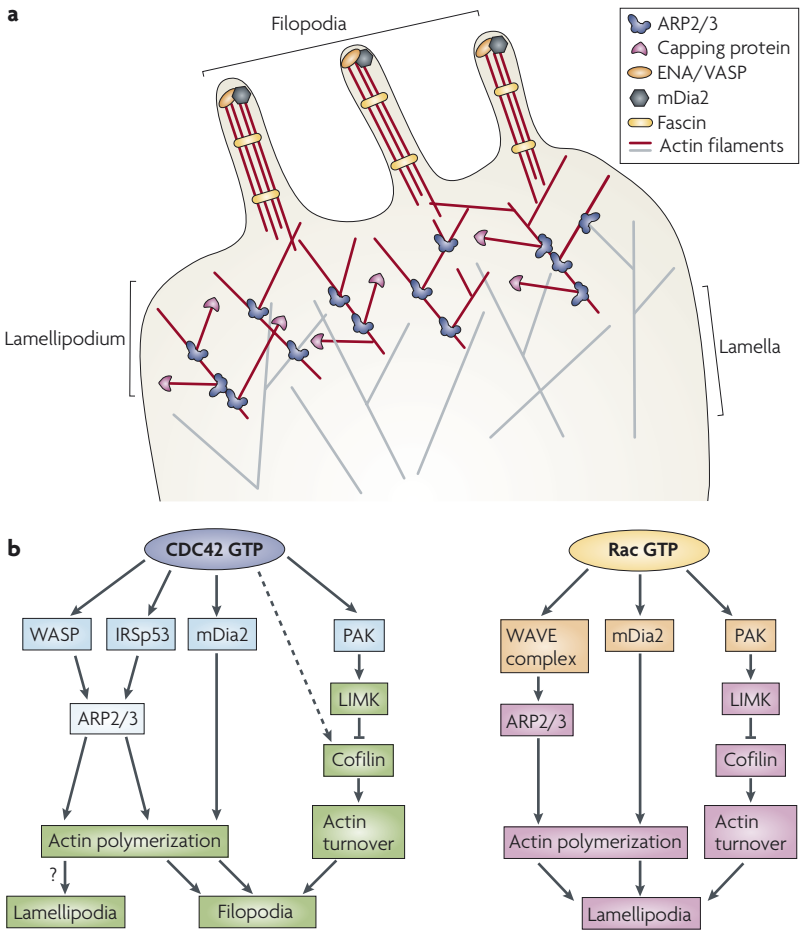


Figure 2 | **Formation of lamellipodia and filopodia. a** | At the leading edge of the cell, the highly dynamic lamellipodium is extended by actin-related protein-2/3 (ARP2/3)-complex-mediated formation of new actin filaments from the sides of existing filaments. This leads to the assembly of a dendritic network of branched actin filaments. Capping proteins bind to the barbed ends to terminate elongation of the actin filaments. Rac activates actin polymerization during lamellipodium formation through the Wiskott–Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE) complex, which activates ARP2/3, and possibly the formin mammalian diaphanous-2 (mDia2), which nucleates unbranched actin filaments. The lamella is located behind the lamellipodium and extends back into the cell body. In the lamella, the actin filaments are longer and less branched, and actin dynamics are thought to be independent from those in the lamellipodium[150]. Filopodia are thin protrusions that contain parallel bundles of actin filaments that extend from the leading edge in many migratory cells and probably function as sensory probes or in the establishment of cell–cell contacts. **b** | CDC42 induces actin polymerization by binding to WASP, the related N-WASP, or through the insulin-receptor substrate p53 (IRSp53) Tyr kinase to induce branched actin filaments using the ARP2/3 complex. Whether this contributes to filopodium extension is unclear. Rac activates the ARP2/3 complex through the WAVE complex. CDC42 and Rac also induce actin polymerization by activation of mDia2. Rac-mediated or CDC42-mediated activation of the Ser/Thr kinase PAK (p21-activated kinase) phosphorylates LIM kinase (LIMK), which phosphorylates and inhibits cofilin, thereby regulating actin-filament turnover. In the neuronal growth cone, CDC42 might result in reduced cofilin phosphorylation by an unknown mechanism (dotted line), thereby stimulating actin polymerization and filopodium formation. ENA/VASP, enabled/vasodilator-stimulated phosphoprotein.

function independently of the PAR complex through its target, myotonic-dystrophy-kinase-related CDC42-binding kinase (MRCK), to move the nucleus behind the MTOC[41]. CDC42 and the PAR complex have been identified in a recent genome-wide screen for regulators of endocytic traffic, which indicates that this pathway could be important for targeting recycling endosomes to specific intracellular sites[42].

The contribution of CDC42 and the PAR complex to epithelial cell polarity has been studied extensively[5,43]. Epithelial cells have a polarized morphology that is defined by an asymmetrical distribution of proteins, which form distinct apical and basal domains. This polarization is important for cell function, allowing directed transport across the epithelial layer while acting as a barrier to small solutes and ions. Adherens junctions provide strong cell–cell contacts, whereas tight junctions control the permeability of the epithelial layer and separate the membrane domains which, together with directed transport, are important in the maintenance of epithelial polarity. Constitutively active and dominant-negative CDC42 affect tight-junction formation and the polarized trafficking of proteins to the apical and basal domains[43,44]. The localization of PAR6–aPKC to the apical domain is dependent on CDC42 and is required for targeting of apical proteins[45].

In vivo studies have confirmed the central role of aPKC downstream of CDC42 in epithelial polarity. For example, in early development, CDC42-null embryoid bodies have defects in both epithelial polarity and in cell contacts, and also have reduced aPKC phosphorylation[35]. In addition, the apical localization of aPKC, PAR6, E-cadherin and β-catenin is abolished following CDC42 loss in the telencephalic neuroepithelium of the brain. Conditional knockout of CDC42 in the epidermis leads to reduced aPKC phosphorylation and gradual loss of cell–cell contacts, which the authors speculate is a consequence of reduced β-catenin levels[46]. Disruption of basement membrane deposition by the basal layer of epidermal keratinocytes is also observed in this conditional model[47], and this is consistent with a role for CDC42 in polarized secretion in epithelial cells[5]. It will be interesting to know whether the PAR complex contributes to these changes of the basement membrane.

Cell fate determination. The analysis of *Cdc42*-knockout mice has demonstrated that regulation of polarity by CDC42 *in vivo* is important in the fate determination of several different cell types following cell division. For example, CDC42 is preferentially localized in neural progenitor cells at the apical ventricular zone (VZ) but not the basal subventricular zone (SVZ) of the neuroepithelium and is required for the apical localization of adherens junctions and the PAR complex[48,49]. Division of a VZ cell normally produces a progenitor cell and a neuron, whereas SVZ-cell division produces two neurons. In *Cdc42*-knockout mice, the loss of PAR-complex localization is associated with a change in fate determination of the VZ cells and the gradual conversion of VZ progenitors to basal SVZ cells, together with an increase in neuron generation[48]. Conditional knockout of CDC42 in the mouse epidermis also affects fate determination and indicates a role for CDC42 in the degradation of β-catenin: loss of CDC42 resulted in differentiation of epidermal keratinocytes in place of hair follicle cells, an

effect that is probably the result of decreased aPKC activity and the subsequent reduction in levels of β-catenin[46]. Finally, conditional knockout of CDC42 in the bone marrow causes defects in multiple haematopoietic lineages, with suppressed erythropoiesis but increased myelopoiesis[50]. It will be interesting to know whether these effects are due to altered PAR-complex localization and subsequent changes in cell fate.

Rac family

Based on sequence similarity, RAC1, RAC2, RAC3 and RhoG form a subfamily within the Rho GTPases[4] (FIG. 1). Rac proteins stimulate lamellipodium and membrane-ruffle formation, and induce membrane extension during phagocytosis[1]. RhoG also stimulates lamellipodium extension and contributes to phagocytosis, probably primarily by activating the Rac GEFs, dedicator of cytokinesis proteins (DOCKs)[51].

The three Rac isoforms have different expression patterns and, despite their high sequence similarity, studies from knockout mice indicate that they have non-redundant functions. *Rac1* is the best-studied member of this family and is ubiquitously expressed, whereas *Rac2* expression is mostly restricted to cells of haematopoietic origin[52,53] and *Rac3* mRNA is most abundant in the brain[54–56]. RhoG, which has the lowest sequence identity to RAC1, is widely expressed, albeit at varying levels within human tissues[57]. *Rac1*-knockout mice are embryonic lethal and show a range of defects in germ-layer formation[58], and thus tissue-specific knockouts have been widely used to study RAC1 function (Supplementary information S1 (table)). *Rac2*-, *Rac3*- and *RhoG*-knockout mice do not show obvious developmental defects, but they do have cell-type-specific functional defects.

Lamellipodium extension and the actin cytoskeleton. Dominant-negative RAC1 inhibits lamellipodium extension, membrane ruffling and migration in multiple cell types, including macrophages, T cells, epithelial cells and fibroblasts[59]. Surprisingly, RAC1-null macrophages can form membrane ruffles and migrate at a similar speed to wild-type macrophages. However, they have an elongated morphology and do not spread normally, which implies that they are defective in lamellipodium extension[60]. Similarly, RAC1-null Schwann cells, endothelial cells and platelets have impaired lamellipodium formation[61–63]. By contrast, RAC1-null neutrophils have multiple lamellae, and although it is not required for migration, RAC1 is required for efficient polarization and chemotaxis towards the chemotactic peptide fMLP[64]. These effects probably reflect the high levels of RAC2 in neutrophils — indeed, RAC2 is essential for formation of lamellipodia and migration of neutrophils[65]. However, in HSC-derived neutrophils, RAC1 might compensate for the loss of RAC2 as RAC1- RAC2-null HSC-derived neutrophils had a stronger reduction in chemotaxis than RAC2-null cells alone[66]. In macrophages, RAC1 is the most abundant isoform[60,67], and RAC2 has only a minor effect on migration, which is dependent on the substratum composition[68]. The ability of RAC1- RAC2-null macrophages to migrate at a similar speed or faster than wild-type cells indicates that these cells use a Rac-independent mechanism to extend protrusions. It would therefore be interesting to test whether other Rho GTPases, such as RhoG, affect macrophage migration. However, RhoG is not required for neutrophil chemotaxis[69].

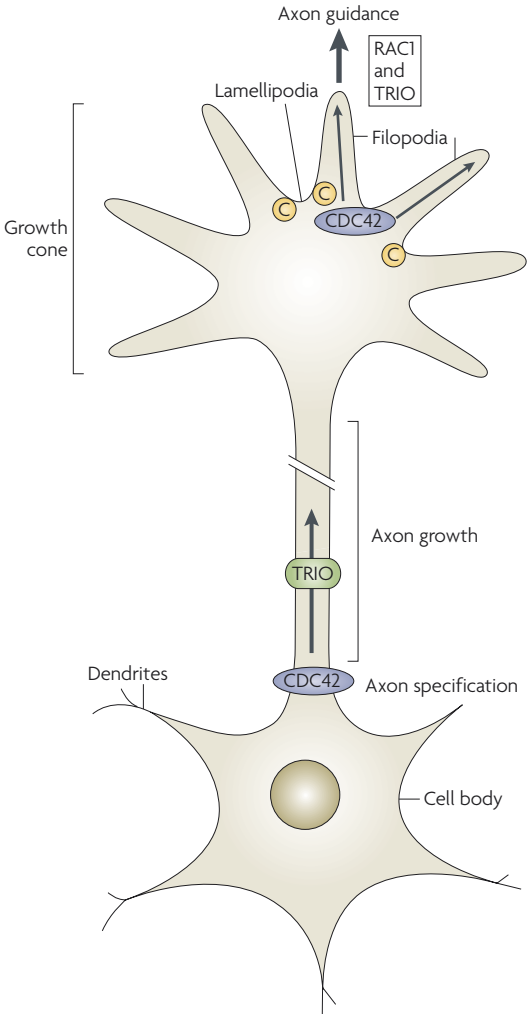


Figure 3 | **Roles of Rho GTPases in neuronal development.** During neuronal development, the axon extends from the cell body over long distances to form synaptic contacts with other cells. At the front of the axon is the growth cone, which is composed of filopodia that are interconnected by lamellipodia. CDC42 is required for the formation of filopodia, as CDC42-null neurons have a reduction in both the number and growth of filopodia. In addition, CDC42 is required for axon specification and extension as CDC42-null neurons have a defect in axonal growth. In wild-type neurons, active cofilin (C) is present in the growth cone, where it is thought to regulate axonal growth and filopodia. CDC42-null neurons have an increase in inactive, phosphorylated cofilin[13]. RAC1 seems to regulate axon guidance, as RAC1-null telencephalic neurons are defective in migration *in vivo*[77]. Similarly, neurons that lack triple functional domain protein (TRIO), a Rho guanine nucleotide-exchange factor for Rac, RhoG and RhoA, show impaired axon guidance and impaired axon extension[78].

Myelin
Multiple layers of plasma membrane made by glial cells that wrap around axons and electrically insulate them. Myelin contains high levels of glycolipids and myelin-specific proteins.

Embryoid bodies
Aggregates of embryonic stem cells that are used to model the early steps of peri-implantation embryonic development, including establishment of epithelial polarity.

In vivo evidence for an important role of Rac-driven lamellipodium extension is suggested by studies on Schwann cells. Schwann cells extend lamellae into axon bundles before myelination. RAC1-null Schwann cells, or cells that have been treated with a Rac inhibitor, do not generate lamellipodia *in vitro* and have a defect in the extension of processes that are required to envelop and myelinate axons *in vivo*[27,61].

Rac proteins regulate actin polymerization during lamellipodial extension in several ways (FIG. 2b). First, they can activate actin-nucleating proteins, including the ARP2/3 complex (through WASP-family verprolin-homologous protein (WAVE) proteins) and mDia formins[1]. Indeed, lamellipodium formation is inhibited in WAVE-null cells[70]. Second, Rac proteins can affect the availability of free actin barbed ends through the removal of barbed-end capping proteins or the severing of actin filaments through cofilin or gelsolin[1]. Third, they can increase the availability of actin monomers for incorporation into actin filaments by regulating cofilin[23]. Recent work in leukocytes from RAC1- and RAC2-null mice has, for the first time, shown that RAC1 and RAC2 have different roles in these processes. In neutrophils, RAC1 and RAC2 are required for the production of free barbed ends using two different pathways: RAC1 induces the uncapping of existing actin filaments, whereas RAC2 functions through the activation of cofilin and the ARP2/3 complex[71]. RAC2 has a dominant effect on actin polymerization[64,72], and similarly F-actin assembly seems to be predominantly controlled by RAC2 in HSC/P cells. By contrast, actin polymerization, lamellipodium extension and ruffling that is induced in macrophages by the cytokine colony-stimulating factor-1 is unaffected by RAC2 deletion[67], which indicates that the contribution of RAC2 to acute actin polymerization depends on the cell type and presumably on levels of RAC2.

Although most Rac studies have concentrated on F-actin in lamellipodia, analysis of erythrocytes has recently shed light on the regulation of the membrane-associated actin cytoskeleton by Rac. A hexagonal lattice of actin filaments crosslinked by spectrins underlies the erythrocyte plasma membrane, giving strength and flexibility to withstand shear forces in circulation. Erythrocytes that lack RAC1 and RAC2 have defects in the actin meshwork and spectrin scaffold, which results in decreased deformability and anaemia *in vivo*[73]. It will be interesting to determine whether this function of Rac proteins is mediated by any of the known Rac targets implicated in lamellipodium extension.

Axon growth versus axon guidance. Similar to CDC42, RAC1, RAC3 and RhoG promote neurite outgrowth, but the relative contribution of RAC1 to axon growth and guidance seems to be variable[74]. In *D. melanogaster*, RAC1 mutants disrupt axon growth[75] and in rat cortical cells dominant-negative RAC1 decreases axon growth[76]. However, studies in a conditional knockout of RAC1 in neurons of the telencephalon showed normal axon outgrowth but failure of axons to cross the midline, which implies that RAC1 is important for axon guidance rather than growth[77]. A related phenotype is observed in mice that lack the RhoGEF TRIO (triple functional domain protein), in which axon guidance is similarly impaired[78]. Axon growth is also reduced in TRIO-null neurons. This effect was proposed to be due to the loss of RAC1 activation, even though TRIO has two GEF domains that can activate Rac, RhoG and RhoA. It is therefore likely that the effect of TRIO might be a combination of Rac, RhoG and RhoA signalling. Indeed, dominant negative RhoG inhibits TRIO-induced neurite outgrowth *in vitro*[79], and constitutively active RhoG in cultured neurons induces sprouting of new axons independent of RAC1 or CDC42 (REF. 80).

It is possible that RAC3 could compensate for RAC1 deletion in axon outgrowth. Overexpression of RAC3 but not RAC1 was reported to increase neurite extension and branching *in vitro*[81], and thus it was surprising that *Rac3*-knockout hippocampal neurons have normal morphology and polarity in culture, and that RAC3-null mice have no gross anatomical defects in brain structure or in the organization of neurons[56]. Behavioural studies revealed hyperactivity to novel stimuli and changes in motor learning[56,82], which suggests that there is a more subtle effect of RAC3 on neuron function. RAC3 might affect the formation of dendritic spines, which receive synaptic input from axons[83]. Dominant-negative RAC1 reduces the formation of dendritic spines in rat cortical neurons *in vitro*[76], and there were more but smaller dendritic spines in mice that express constitutively active RAC1 in Purkinje cells[84]. Interestingly, RNA interference (RNAi)-mediated depletion of RAC1 in RAC3-null hippocampal neurons inhibited dendrite formation but not axon formation[85]. This is consistent with a model in which RAC1 and RAC3 contribute to axon guidance rather than to axon initiation or outgrowth. Given that CDC42 is important for axon growth (see above), dominant-negative RAC1 might affect axon growth by altering CDC42 activity rather than RAC1 activity.

Adhesion and differentiation. The formation and turnover of cell–cell and cell–substratum adhesions is important in leukocytes for adhesion to the blood vessel wall and for recruitment to and retention in specific tissues, and in epithelial cells for the regulation of epithelial integrity and differentiation. In T cells, dominant-negative RAC1 has previously been shown to inhibit chemokine-induced adhesion to integrin ligands[86], although Rac has been postulated to function primarily by stimulating spreading rather than regulating the expression or affinity of integrins[87]. Analysis of Rac-null cells has revealed that both RAC1 and RAC2 are important for regulating adhesion-induced spreading. Loss of RAC2 in macrophages or neutrophils leads to selective defects in integrin-mediated spreading and migration without affecting adhesion[65,88]. RAC1-null macrophages also have reduced spreading[60], as do RAC1-null MEFs[89]. In RAC1-null MEFs, this defect seems to lead to enhanced apoptosis. RAC2-null neutrophils have attachment defects through the adhesion receptor L-selectin, which is important for leukocyte

Barbed ends
The fast-growing ends of actin filaments, so-called because of their appearance in electron micrographs following binding of a fragment of myosin.

Capping proteins
Proteins that bind to the ends of actin filaments and prevent actin polymerization.

Spectrins
A family of large, mostly α-helical proteins that form a plasma-membrane-associated lattice that consists of spectrin tetramers and short actin filaments.

capture by endothelial cells in the bloodstream[65]. Differences in adhesion responses and subsequent transendothelial migration could be responsible for the reduced accumulation of neutrophils and macrophages that lack RAC1 or RAC2 in an inflammatory peritoneal model *in vivo*[65,67,90].

Studies using HSC/P cells suggest that RAC1 and RAC2 have different roles in adhesion processes. HSC/P cells normally reside in the bone marrow and generate the progenitor cells of the immune system. RAC2-null HSC/P cells show defective adhesion to integrin substrates and to a bone-derived stromal cell line, which suggests that their reduced accumulation and retention in the bone marrow is a consequence of decreased adhesion[37,91]. RAC1-null HSC/P cells are strongly impaired in homing to the stem-cell niche in the bone marrow, whereas transendothelial migration and initial entry into the bone marrow are not affected[66,92]. The signalling pathways that are activated by RAC1 and RAC2 in HSC/P cells to affect their adhesion in the bone marrow are unknown.

Rac isoforms and phagocytosis. Phagocytosis is the process by which bacteria, apoptotic cells and other particles are engulfed. Antibody-coated particles are phagocytosed through Fc receptors (FcRs), whereas complement receptors, such as CR3 (integrin αMβ2), phagocytose bacteria by antibody-dependent and independent methods. Actin polymerization and the ARP2/3 complex are required for particle engulfment[93–95]. Studies using dominant-negative Rho GTPases have indicated that different Rho GTPases contribute to phagocytosis depending on the receptor: CDC42 and Rac are required for FcR-mediated uptake and apoptotic cell phagocytosis, whereas Rho is required for complement-mediated phagocytosis[96,97]. Dominant-negative RhoG inhibits phagocytosis of apoptotic cells[98], and could function upstream of RAC1 (REF. 99).

The involvement of Rac isoforms in phagocytosis has been investigated further with cells from knockout mice. RAC2-null neutrophils have a moderate inhibition of phagocytosis of serum-coated bacteria, whereas loss of RAC1 has no effect[100]. Neutrophils from a human patient with a dominant-negative mutation in RAC2 also have impaired FcR-mediated phagocytosis[101]. RAC2-null macrophages are defective for uptake of antibody-coated particles but not serum-coated zymosan. As zymosan can bind to both FcR and CR3, this suggests that RAC2 is not required for CR3 signalling in macrophages. Actin polymerization that is induced in response to FcR-mediated phagocytosis in macrophages was not affected by loss of RAC2 (REF. 67). This is consistent with analysis of RAC1-, RAC2- and CDC42-activation kinetics in macrophages, which indicates that RAC1 and CDC42 are most likely to regulate actin polymerization, whereas RAC2 is activated in an F-actin-depleted region at the base of the phagocytic cup[102]. Consistent with a model in which each Rac isoform has a different function, macrophages that lack both RAC1 and RAC2 are defective for both FcR and CR3-mediated phagocytosis[103].

Taken together, the current data indicate that RAC2 but not RAC1 is important in FcR-mediated phagocytosis in neutrophils, whereas the potential contribution of Rac isoforms in macrophages and in apoptotic cell and complement-mediated phagocytosis deserves further investigation.

NADPH oxidase and bacterial killing. During phagocytosis, neutrophils and macrophages generate reactive oxygen species (ROS) by the localized recruitment and activation of the NADPH oxidase complex on the phagosome membrane. The NADPH oxidase reduces oxygen to superoxide, which then contributes to bacterial killing[104]. Both RAC1 and RAC2 were identified in cell-free systems as components of the NADPH oxidase complex and contribute to complex assembly and activation[105]. However, subsequent studies using knockout mice have identified only RAC2 as the Rac isoform that is involved in superoxide generation in leukocytes. Neutrophils and macrophages from RAC2-null mice have defective superoxide production[65,67,106], despite compensatory increases in RAC1 activity[72]. Neutrophils from a human patient with a dominant-negative mutation in RAC2 also demonstrated defective superoxide production[101]. By contrast, NADPH oxidase activity is unaffected in RAC1-null neutrophils[90]. Analysis of RhoG-null neutrophils revealed a new role for RhoG in regulating NADPH oxidase activation; loss of RhoG is associated with a reduction in RAC1 and RAC2 activation, but whether this effect is responsible for the defect in NADPH oxidase activity is unknown[69].

Bacterial killing is not only dependent on NADPH oxidase activity, but also on the secretion of granules that contain enzymes to damage bacteria. RAC2 is required for granule release, whereas RAC1 has only a minor role in this process[100,107]. These findings suggest that RAC2 coordinates both granule release and NADPH oxidase activation during phagocytosis.

The predominance of RAC2 in NADPH oxidase activation is likely to be restricted to leukocytes, as RAC1-null cardiomyocytes (which do not express RAC2 or RAC3) have reduced NAPDH oxidase activity[108], and RAC1-null osteoclasts have lower ROS levels even though they express RAC2 (REF. 109). RAC1-null embryo fibroblasts, however, have higher ROS levels as a consequence of RAC3 upregulation. This leads to increased DNA damage — a known consequence of high ROS levels — and subsequent cell senescence[110]. These results could implicate Rac isoforms in ROS-induced DNA damage in human diseases, for example cancer[111].

Rho proteins

The three Rho isoforms — RhoA, RhoB and RhoC — are highly homologous (FIG. 1), and all induce stress-fibre formation when overexpressed in fibroblasts[112]. The functions of Rho in cellular responses have been investigated extensively using the clostridial enzyme C3 transferase, which modifies and inhibits all three isoforms. Dominant-negative RhoA, RhoB and RhoC have also been studied and have clearly distinguishable

Phagocytic cup
Plasma membrane extension around a particle that is in the process of being engulfed by phagocytosis.

effects in cells[113], although whether they inhibit different RhoGEFs (BOX 1) is unknown. RhoB-null and RhoC-null mice are viable and have no major developmental defects[114,115], and studies of specific cell types imply that RhoB and RhoC have different functions *in vivo* (Supplementary information S1 (table)), although they have not been directly compared. *Rhoa*-knockout mice have not been reported. Studies of mice and cells that lack RhoB and RhoC have focused on the contribution of these proteins to vesicle trafficking and to cancer development.

Control of endosome trafficking. Following their internalization, receptors are sorted by trafficking through endocytic compartments. RhoB localizes to endocytic vesicles and regulates endocytic trafficking[116,117]. For example, dominant-negative and constitutively active RhoB alter epidermal growth factor (EGF) receptor endocytic trafficking and transcytosis of endosomes from basolateral to apical membranes in polarized epithelial cells[113,118,119]. Similarly, RhoB-null vascular smooth muscle cells have impaired endosomal trafficking of platelet-derived growth factor receptor (PDGFR-β) following PDGF stimulation; lack of RhoB caused impaired PDGF-induced signalling and proliferation[120], which is consistent with a model in which growth factor receptors signal in part from endosomes[121].

Although RhoB-null mice are viable and fertile, they are smaller than wild-type mice[114]. This phenotype could reflect a defect in angiogenesis, as vascular development of the retina was delayed[122]. Using a combination of farnesyl transferase inhibitors (which affect RhoB lipid modification) and dominant-negative RhoB and morpholinos against RhoB, loss of RhoB was shown to cause apoptosis in primary endothelial cells during sprouting angiogenesis *in vivo* and endothelial tube formation *in vitro*. RhoB affected the nuclear trafficking of the kinase AKT/protein kinase B in endothelial cells, a mechanism that is postulated to regulate endothelial cell survival[122]. This mechanism might reflect alterations in trafficking and signalling by the vascular endothelial growth factor (VEGF) receptor.

Cancer progression and metastasis. Expression studies in human cancers and *in vitro* and *in vivo* cancer models show that RhoB and RhoC seem to have different roles in cancer progression. RhoB is proposed to function as a tumour suppressor as its expression is reduced in tumours and its overexpression inhibits cell growth and survival, invasion and metastasis[123,124]. RhoB-null mice have increased susceptibility to carcinogen-induced skin tumours[114]. Neoplastically transformed RhoB-null MEFs have defects in adhesion and spreading, and are more sensitive to transforming growth factor-β (TGFβ)-induced changes in the actin cytoskeleton and proliferation[114]. This is consistent with evidence that RhoB regulates TGFβ receptor expression[125]. It will be interesting to know whether these properties contribute to cancer development *in vivo* and whether they reflect changes in endosomal trafficking of receptors.

The expression of RhoC correlates with metastasis for several cancer types[124,126] and is sufficient to induce metastasis in poorly metastatic cells[127]. Dominant-negative RhoC or RNAi-mediated RhoC knockdown reduces the migration and invasion of prostate, gastric and breast cancer cells[128–130]. In a lung metastasis model, RhoC is not required for tumour initiation, but RhoC-null mice have a much reduced number and size of metastases, which correlates with a decrease in metastatic cell survival, tumour cell motility and invasion[115]. A mechanism for the upregulation of RhoC in breast cancer metastasis was recently reported. The microRNA *mir-10b* was found to be expressed in metastatic breast cancers and was reported to indirectly induce the expression of RhoC through inhibition of the transcription factor homeobox protein D10 (HOXD10). Expression of *mir-10b* stimulates invasion *in vitro* and metastasis *in vivo* through RhoC[131]. The mechanism by which RhoC increases invasion and metastasis is not yet understood, although it might reflect its ability to activate Rho-associated kinase (ROCK). ROCK stimulates actomyosin-based contractility and the loss of cell–cell junctions and induces the invasion of colon tumour cells *in vivo*[132,133].

RhoH

The *RhoH* gene evolved later than other Rho GTPases and is only present in vertebrates[4]. It is predominantly expressed in haematopoietic cells, and RhoH-null mice are viable and fertile[134]. RhoH has low or no GTPase activity and is thus constitutively bound to GTP in cells[135,136]. Its expression is regulated by transcription[136] and alternative 5′ splicing[137].

RhoH was initially proposed to inhibit signalling by other Rho GTPases, as overexpression of RhoH inhibited RAC1- and RhoA-mediated activation of the transcription factor nuclear factor-κB (NFκB) and p38 MAPK signalling in Jurkat cells[136]. Overexpression of RhoH specifically reduces Rac activity in HSC/P cells and attenuates Rac-dependent responses, including chemokine-induced cortical F-actin assembly and migration[138]. Consistent with these findings, RhoH-null T cells and HSCs show hyperactivation of RAC1 but not RAC2, and increased migration and chemotaxis[139,140]. Knockdown of RhoH by RNAi in human lymphocytes results in the activation of the integrin leukocyte cell-surface antigen-1 (LFA1)[141]; this effect might be a consequence of increased Rac activity, which can activate LFA1 (REF. 142). However, binding to the LFA1 ligand intercellular adhesion molecule-1 (ICAM1) is unaffected in RhoH-null T cells[139] and thus it is possible that modulation of LFA1 activity by RhoH is dependent on T-cell differentiation state. RhoH inhibits Rac recruitment to the plasma membrane, but it is unknown how RhoH regulates Rac activity at a molecular level (FIG. 4a).

Analysis of *RhoH*-knockout mice has revealed a previously unknown function for RhoH in T-cell signalling[134,139]. RhoH-null mice have defects in T-cell maturation owing to reduced T-cell receptor (TCR) signalling, and hence have lower numbers of T cells (FIG. 4).

Farnesyl transferase
An enzyme that adds a 15-carbon isoprenoid called a farnesyl group to a Cys residue near the C terminus of a number of proteins, including several Rho GTPases. Other Rho GTPases, such as RhoA and RAC1, are modified by the addition of a 20-carbon geranylgeranyl group.

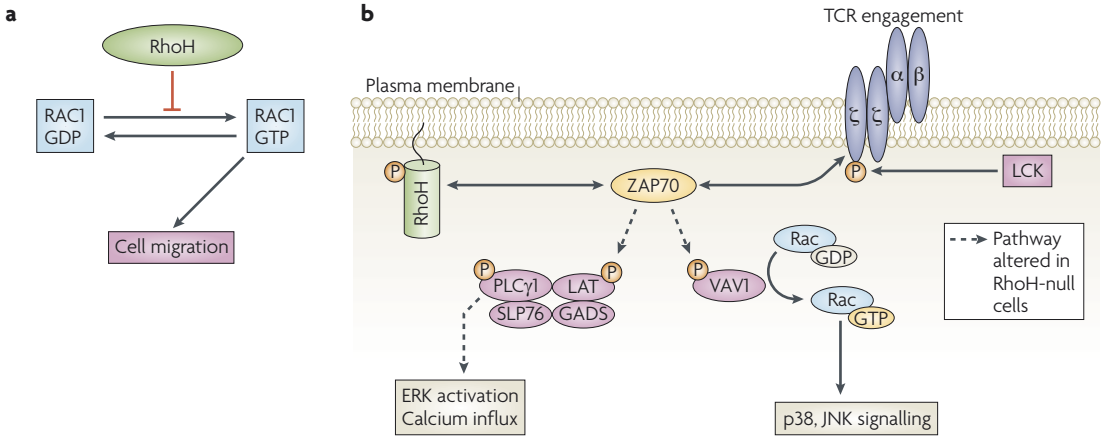


Figure 4 | **Roles of RhoH in T cells. a** | Overexpression of RhoH reduces Rac activity[138], whereas loss of RhoH increases the basal level of RAC1 (REF. 139). This implicates RhoH in the control of RAC1 activation by an unknown mechanism, either through the regulation of a guanine nucleotide-exchange factor (GEF) or a GTPase-activating protein (GAP). **b** | T-cell receptor (TCR) engagement leads to the activation of the Tyr kinase LCK. LCK-mediated Tyr phosphorylation of cluster of differentiation-3ζ (CD3ζ) then serves as a docking site for ζ-chain-associated protein kinase-70 (ZAP70)[134]. Subsequent downstream signalling from activated ZAP70 includes phosphorylation of the adaptor protein LAT (linker for activation of T cells) and phospholipase Cγ1 (PLCγ1) and results in a variety of responses, including calcium influx, cell proliferation and changes in gene expression[151]. RhoH was shown to interact with ZAP70, and this interaction seems to be mediated by LCK-induced Tyr phosphorylation of RhoH[134]. ZAP70-mediated Tyr phosphorylation of LAT and phosphorylation of PLCγ1 was reduced in RhoH-null T cells. Downstream of TCR signalling, PLCγ1-induced extracellular signal-regulated kinase (ERK) activation and calcium influx were also inhibited in RhoH-null cells. In addition, phosphorylation of the RacGEF VAV1 was reduced but RAC1 and RAC2 activity was not altered[139]. The data suggest a model in which phosphorylated RhoH is required for recruitment of ZAP70 to the TCR and for subsequent signalling downstream of ZAP70 following TCR engagement. GADS, GRB2-related adaptor downstream of Shc; JNK, Jun N-terminal kinase; SLP76, SH2 domain-containing leukocyte protein of 76 kDa.

RhoH was found to interact directly with the Tyr kinase ζ-chain-associated protein kinase-70 (ZAP70), which is part of the TCR signalling cascade and crucial for T-cell development. The interaction between RhoH and ZAP70 was strongly enhanced by Tyr phosphorylation of RhoH. The data suggest a model in which TCR-induced RhoH phosphorylation leads to RhoH-dependent recruitment of ZAP70 through its Src-homology-2 (SH2) domains to the plasma membrane[134], although it is controversial whether RhoH affects Tyr phosphorylation of ZAP70, which is usually mediated by LCK[134,139] (FIG. 4b). ZAP70 normally binds to the Tyr phosphorylated TCRζ subunit[143] (FIG. 4b), and it is possible that RhoH facilitates this interaction. RhoH would thereby contribute to TCR signalling, including phosphorylation of the Rac-exchange factor VAV1 (REF. 139). Surprisingly, despite this effect on VAV1, TCR-induced Rac activation is not affected by RhoH[139], in contrast to the observed RhoH-induced decrease in basal Rac activity in T cells described above. It will therefore be interesting to determine how RhoH normally affects Rac activity, and why this does not occur in TCR signalling.

Although the function of RhoH in HSC and T cells is beginning to be elucidated, its role in other haematopoietic cells remains unclear. RhoH is expressed in myeloid and lymphoid cells and at high levels in a B-cell line[136,138], but unlike T cells, B-cell numbers and differentiation seem normal in *RhoH*-knockout mice[134,139]. This suggests that RhoH does not regulate B-cell development.

Conclusions and future directions

Since the initial identification of the functions of Rho and Rac in regulating the actin cytoskeleton, many studies in cultured cell lines over the past 17 years have described a variety of functions for individual Rho GTPases, predominantly by expressing constitutively active and dominant-negative mutants. In addition, biochemical studies have found connections between GEFs, GAPs and Rho GTPases, and identified a plethora of downstream targets for Rho GTPases. Analysis of mice that lack some of the Rho GTPases in specific tissues and analysis of cells derived from these knockout mice has rapidly generated a wealth of information on how these proteins affect developmental processes and cell behaviour. This would be impossible to analyse in tissue-culture models. In many cases, results with knockout mice correlate with those observed *in vitro* with dominant-negative mutants, providing reassurance that cell-culture models are useful and informative. However, in other cases, analysis of cells from knockout mice gives clearly different or even opposite effects to what has been predicted from dominant-negative mutant studies. Some of these differences could be due to functional redundancy between closely related Rho isoforms — indeed, upregulation of other Rho GTPases is observed in a number of knockout models. Clarifying why there are differences in signalling between loss of a gene and expression of a dominant-negative mutant will be an important goal for the future.

Studies on knockout mice have also allowed a careful comparison between closely related isoforms; for example, among the Rac isoforms, RAC1, RAC2 and RAC3. As most scientists working with *in vitro* systems are now using RNAi to knockdown Rho GTPase expression instead of using constitutive active or dominant-negative mutants, it will be interesting to compare RNAi results with those from knockout mice.

So far knockouts of only 7 of the 20 Rho GTPase family members have been described. In the future, it will be important to generate knockouts of the other family members to resolve controversies such as whether CDC42 is required for filopodium extension[9]. Like the *Rac1* and *Cdc42* knockouts, it is likely that knockout of *Rhoa* will be lethal, and thus that a conditional knockout of *Rhoa* will be needed to understand how this protein functions in development.

In the future, more detailed analysis of the links between each Rho GTPase and its interacting partners will be crucial to providing a molecular understanding of Rho GTPase-driven cellular responses as well as developmental processes. Importantly, knockouts of several of the many RhoGEFs, RhoGAPs and Rho GTPase targets have been made, and thus it will be useful to compare the phenotype of these mice with Rho GTPase-null mice. These mice also provide models for human diseases, such as the CDC42GEF faciogenital dysplasia-1 (FGD1) in facio-cranial syndrome, and the RhoGAP oligophrenin in mental retardation.

Given the new insight provided from detailed analysis of the Rho GTPase knockouts so far, we can expect a plethora of information on RhoA and other Rho GTPases from mouse models in the near future.

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Acknowledgements
We apologize to all those authors in the field whose papers we could not cite because of space limitations. We thank members of our laboratory for discussions. Work in our laboratory is supported by the Medical Research Council UK, Cancer Research UK and the European Commission Network of Excellence, MAIN.

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