Chapter 3
Regulation of the Cytoskeleton by the Rho Family of GTPases in Hematopoietic Stem Cells in Health and Disease

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Introduction

Rho-family GTPases comprise a main branch of the Ras superfamily of small GTPases. Twenty-two human members of the Rho family have been identified, and can be subdivided into ten groups on the basis of their identity to Cdc42, Rac1, RhoA, RhoD, Rif/RhoF, Rnd3/RhoE, TTF/RhoH, Chp/RhoV, mitochondrial Rho (Miro1/RhoT1), or Rho-related BTB-domain-containing protein (RhoBTB). They are low molecular weight guanine nucleotide binding proteins and function as binary molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state [1]. Rho GTPases are involved in most, if not all, actin-dependent processes such as those involved in migration, adhesion, morphogenesis, axon guidance, and phagocytosis. In classic fibroblast studies, activation of RhoA, Rac1, and Cdc42 led to the reorganization of the actin cytoskeleton into distinct structures: stress fibers and focal adhesions, veil-like lamellipodia, and filopodial microspikes, respectively [2, 3]. In addition to their regulation of the actin cytoskeleton, Rho GTPases have been shown to play vital roles in a number of cellular processes such as the regulation of enzymatic activities, cell adhesion, intracellular signaling cascades, endocytosis, vesicle trafficking, G1 cell cycle progression, oncogenesis, gene transcription, microtubule dynamics, cell polarity, and asymmetric cell division (ACD) [1, 4–14].

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In an earlier review, we described the regulation of the actin and microtubule cytoskeleton by Rho GTPases [15]. The actin and microtubule cytoskeleton mediates a variety of essential biological functions in all eukaryotic cells. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties provide the driving force for cells to move and to divide. Understanding the biochemical mechanisms by which Rho GTPases control the cell cytoskeleton is thus a major goal of cell biology and has implications for human health and disease.

Regulation of Rho GTPase Activity

Rho GTPases are molecular switches and cycle between guanosine triphosphate (GTP)-bound (on) and guanosine diphosphate (GDP)-bound (off) state. Cycling between GTP- and GDP-bound states is controlled primarily by three classes of regulatory molecules: GTPase-activating proteins (GAPs) enhance the relatively slow intrinsic GTPase activity of Rho proteins; guanine nucleotide-exchange factors (GEFs) catalyze the exchange of GDP for GTP; and Rho GTPase guanine nucleotide dissociation inhibitors (Rho GDIs) interact and stabilize the GDP-bound form. GAPs suppress Rho activity, whereas GEFs promote it. In cells, GTP is preferentially loaded into Rho GTPases during nucleotide exchange, because GTP is found at substantially higher concentrations than GDP. Rho GDIs bind to the prenylated forms of Rho-GDP and regulate their distribution between cytosol and the membrane [15]. The number of GEFs (>60) and GAPs (>80) identified so far in the human genome is far more than the number of Rho GTPases (currently 22), suggesting a tight and spatio-temporal regulation of Rho GTPase activity [16–18]. In addition to GEF/GAP regulation, post translational modifications, such as ubiquitination, phosphorylation, isoprenylation, transglutamination, AMPylation, SUMOylation, and oxidation of conserved cysteine residues, critically control the expression and activity of the Rho GTPases [13, 19–22]. A summary of the regulation of Rho GTPases activity is outlined in Fig. 3.1. Rho GTPase expression is also regulated by various micro RNAs (MiRNAs) at the mRNA level [20]. The constitutively active GTP-bound RhoH and Rnd isoforms are regulated by tissue-specific differential expression [23, 24].

The first mammalian GEF, Dbl, isolated in 1985 as an oncogene in an NIH 3T3 focus formation assay using DNA from a human diffuse B-cell lymphoma [25], was found to contain a region of ~180 amino acids that showed significant sequence similarity to CDC24, a protein identified genetically as an upstream activator of CDC42 in yeast [26, 27]. Dbl and Cdc24 represented the initial members of a new family of GEFs. The region of homology among the Dbl family members contains an ~200 residue Dbl homology (DH) domain and an adjacent, C-terminal, ~100 residue pleckstrin homology (PH) domain. The GEFs with the same substrate specificity often have ~20 % sequence identity. The breakpoint cluster region (BCR) was identified as the first RhoGAP isoform, and contains a 150 amino acid GAP domain which is required to induce the GTPase activity of the Rho GTPases [28, 29].

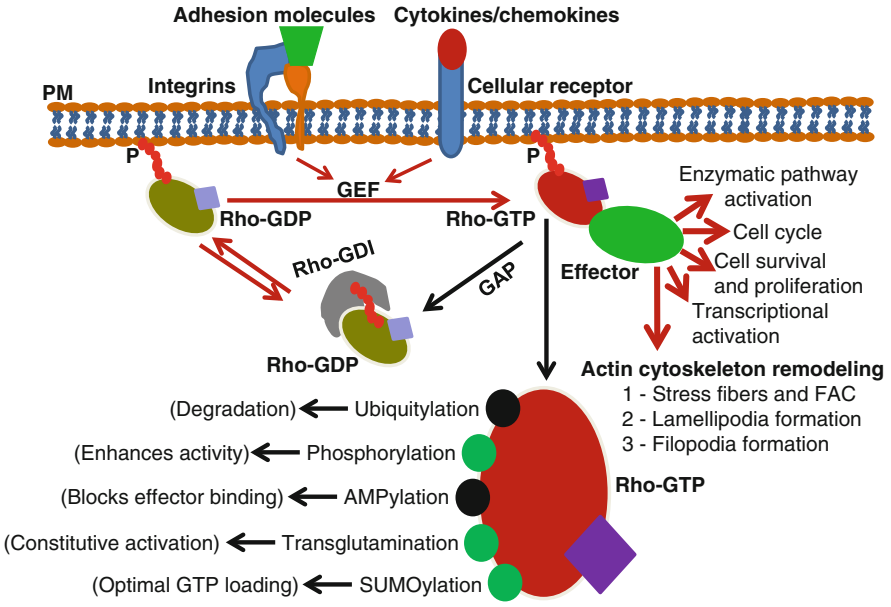

Fig. 3.1 Regulation of Rho GTPase activity by guanine nucleotide-exchange factor/GTPase-activating protein (GEF/GAP) cycling and post-translational covalent modifications. The interaction of cytokines/growth factors and adhesion molecules in the extracellular matrix with their cellular receptors expressed on hematopoietic stem cells (HSCs) triggers the activation of GEFs. GEFs catalyze the exchange of GDP with GTP resulting in Rho GTPase activation. The activated Rho GTPases interact with effector proteins and modulate cytoskeletal rearrangement, leading to the formation of actin stress fibers and focal adhesion contact (FAC), lemellipodia, and filopodia. The activated Rho GTPases also modulate various enzymatic pathways, cell cycle progression, and transcriptional activation, and therefore, regulate a number of cellular processes, such as survival, proliferation, adhesion, migration, differentiation etc. Rho GTPase activating proteins (Rho-GAPs) inactivate Rho GTPase by inducing intrinsic GTPase activity which hydrolyzes bound GTP. Rho GDIs (GDP dissociation inhibitors) bind to the iso-prenylated and GDP-bound form of Rho GTPases and regulate membrane versus cytosolic distribution. Rho GDI also prevents spontaneous activation as well as degradation of GDP-bound Rho GTPases. The post-translational and covalent modifications such as ubiquitination, AMPylation, transglutamination, phosphorylation, and SUMOylation constitutively activate or inactivate the RhoGTPases. *P* = iso-prenylation

Rho GTPase Regulation of HSC Activities

Hematopoietic stem cells (HSCs) are the most actively dividing somatic cells and produce billions of blood cells every day. The two critical features; indefinite self-renewability and multi-lineage differentiation potential, enable the HSCs to maintain their own levels while generating mature blood cells indefinitely. The tight regulation and balance between self-renewal and differentiation potential is critical for steady-state hematopoiesis. During mammalian embryonic development, HSCs

first originate in the extra embryonic yolk sac and the para aortic splanchnopleura/aorta gonad mesonephros (AGM) of the embryo proper. During the prenatal and perinatal period, HSCs migrate to the fetal liver and spleen, and finally to the bone marrow (BM), where they reside and produce all hematopoietic cells in adequate quantity throughout life [30–35]. Various cell types in the BM microenvironment play critical roles in regulating HSC activities during both steady-state and stress hematopoiesis, for example, osteoblasts, endothelial cells, perivascular CXCL12 producing reticular cells, nestin^{+} mesenchymal stem cells, and leptin receptor+ perivascular stromal cells [36–43]. The stromal derived factor (SDF-1α) and stem cell factor (SCF) produced and secreted by BM niche cells, and the fibronectin in the extracellular matrix (ECM), interact with the cellular receptors CXCR4, c-Kit, and integrins, respectively, which are expressed on HSCs, and provide signals for the maintenance of HSC activities [44–51]. Rho GTPases integrate these niche signals into the various cellular processes of HSCs by modulating cytoskeletal rearrangement, gene expression, and the activity of enzymes involved in proliferation and survival pathways. At any given time, a small fraction of hematopoietic stem cells and progenitors (HSC/P) leave the BM, migrate towards peripheral blood (PB), and then migrate back to the BM niche. The migration of HSC/P from the BM niche to the PB is called mobilization, and the migration from the PB towards the BM microenvironment is called homing [52, 53]. Stem cell mobilization and homing are exploited therapeutically for HSC/P transplantation in BM failure and leukemic patients [53–56]. The migration of HSC/P during embryonic development, steady state hematopoiesis, and after stem cell transplantation is critically regulated by Rho GTPases. The multidimensional roles of different Rho GTPases in the regulation of HSC/P activities are depicted in Fig. 3.2.

RhoA Controls the Multilineage Commitment Potential of Hematopoietic Progenitor Cells

RhoA, a member of the Rho family of GTPases, is well characterized in most cell types. Until recently, its role in the activities of HSCs was controversial. Inhibition of RhoA through the retroviral mediated expression of the dominant negative (DN) mutant RhoA N19 has been found to enhance the self-renewal and engraftment potential of HSCs [57]. Another study showed that elevated RhoA activity after deletion of p190-RhoGAP, a negative regulator of RhoA, was advantageous for the self-renewal and long-term engraftment potential of HSC/Ps [58]. The interpretation of these data is affected by the possible off-target effect of the over-expression of the dominant negative mutant form of RhoA in HSC/P. Recently, Zhou et al. [59] conclusively described the functions of RhoA in HSC/P activities using a conditional gene-targeted murine model. The results of this study showed that: RhoA deficiency causes a multilineage hematopoietic failure which is associated with a blockage of hematopoiesis at the multipotent progenitor stage; RhoA$^{-/-}$ HSCs retain long-term engraftment potential, but fail to produce multi potent progenitors and

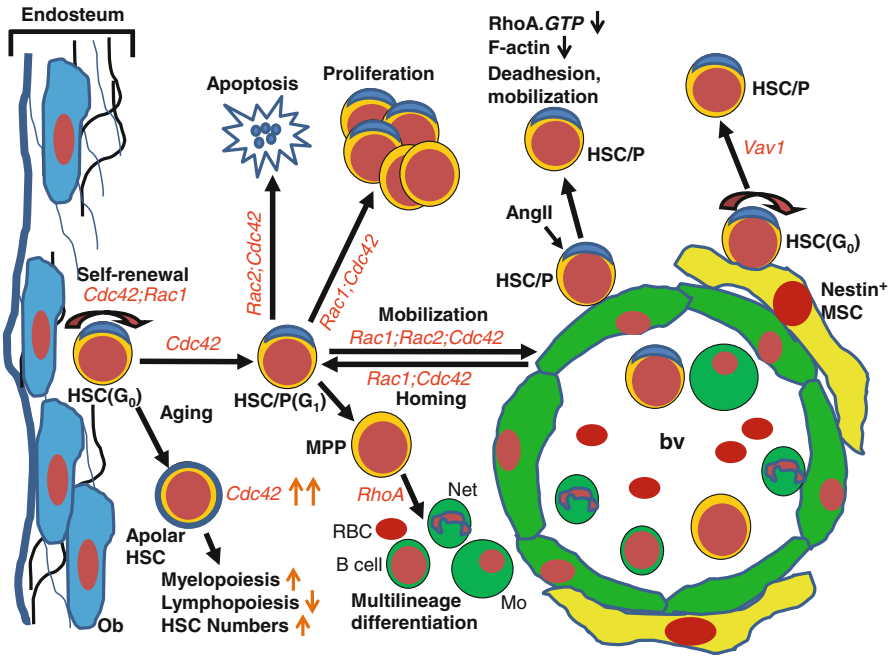

Fig. 3.2 Rho GTPase regulation of hematopoietic stem cell and progenitors (HSC/P) activities. Rho GTPases such as RhoA, Rac (Rac1 and Rac2), Cdc42, and the guanine nucleotide-exchange factor (GEF) Vav, regulate HSC/P functions. Rac1 and Cdc42 control the self-renewal, and Cdc42 regulates the entry of quiescent HSCs into the cell cycle. Rac1 and Cdc42 control HSC/P proliferation, whereas Rac2 and Cdc42 regulate their survival. Rac1 and Cdc42 are required for HSC homing to the bone marrow microenvironment. Rac1, Rac2, and CDC42 play critical roles in the retention of HSC in the bone marrow microenvironment. The elevated level of Cdc42 seen in aged HSC affects HSC polarity, and apolar aged HSCs show a myeloid biased phenotype. RhoA controls the cytokinesis and multi-lineage differentiation of multi potent progenitors (MPPs) leading to formation of mature blood cells. Vav1, a hematopoietic specific GEF, regulates the localization of HSCs near nestin$^+$ mesenchymal stem cells in the perivascular bone marrow niche. An increased level of angiotensin II (Ang II) induces deadhesion of HSC/P from endothelial cells and therefore, enhances HSC/P mobilization to the peripheral blood. Ang II treatment reduces the level of activated RhoA, resulting in a decreased F-actin content in the cortical region of HSC/Ps that eventually leads to deadhesion and mobilization. *bv* blood vessel, *Net* neutrophil, *Mo* monocyte, *HSC* hematopoietic stem cell, *Ob* osteoblast

mature blood cells; RhoA null HSC/Ps show reduced actomyosin-signaling and defective cytokinesis; Defective cytokinesis and programmed necrosis of the hematopoietic progenitors result in a bloodless phenotype; and the loss of RhoA results in a mitotic failure of progenitors manifested by an accumulation of multinucleated cells due to failed cytokinesis and abscission of the cleavage furrow post-telophase, and increased programmed necrosis. Recently, in a hyperangiotensinamia murine model and in sickle cell patients, we have demonstrated that treatment with angiotensin reduces the RhoA activity and actomyosin signaling that leads to de-adhesion of the HSC/P from endothelial cells and mobilization to the PB [146].

Rac GTPases Regulate HSC/P Homing, Retention, and Migration

Rac GTPases modulate actin cytoskeleton rearrangement and F-actin branching, and regulate membrane protrusion, lemellipodia formation, and directional migration in fibroblasts and many other cell types [1]. The SDF-1α, SCF, and epidermal growth factor (EGF) produced and secreted by niche cells, and the fibronectin in ECM interact with the receptors CXCR4, c-Kit, EGFR and integrin, respectively, which are expressed on HSC/P and trigger the activation of Rho GTPases through upstream regulator GEFs. The chemotactic migration of HSC/P towards SDF-1α is mediated by both Rac1 and Rac2, and regulated by the Rac GEF Tiam1 [60]. Rac1 and Rac2 integrate the signals derived from SCF/c-Kit and fibronectin/b1-integrins interactions with various cellular processes, such as homing, migration, and interaction with the microenvironment, and the long-term engraftment potential of HSC/P [61–63]. Initial studies employed Rho GTPase inhibitors (bacterial toxins) to understand the role of Rho GTPases in the regulation of HSC/P activities [64]. However, the specificity of the inhibitors towards individual Rho GTPases was questionable due to a high degree of homology between different isoforms of Rho GTPases (92 % sequence homology between Rac 1 and Rac2). The Rac GTPase subfamily is comprised of three highly homologous isoforms: Rac1, Rac2, and Rac3. Rac1 is ubiquitously expressed, Rac2 is hematopoietic specific, and Rac3 is highly expressed in the central nervous system [65, 66]. Therefore, the non-specific and off-target effects of GTPase inhibitors are highly predictable. Our group has conclusively identified the specific and overlapping functions of the individual Rho GTPase isoforms using gene-targeted mouse models deficient in Rac1, Rac2, Cdc42, RhoH, and specific GAPs and GEFs. Yang et al. studied the role of hematopoietic specific Rac2 using a gene-targeted mouse model. The $Rac2^{-/-}$ mice showed increased numbers of circulating HSC/Ps in the PB due to defective adhesion to the BM microenvironment. HSC/Ps lacking Rac2 were defective in actin cytoskeleton remodeling and α4β1-mediated adhesion to fibronectin or vascular cell adhesion protein 1 (VCAM 1) [67]. Interestingly, the Rac2 null HSC/Ps showed increased migration towards a CXCL-12 (SDF-1α) gradient, possibly through the compensatory upregulation of Rac1 and Cdc42 activities. This study, for the first time, suggested a critical role for Rac2 in the retention of HSC/Ps in the BM microenvironment, and indicated the involvement of cross-talk between Rac and Cdc42. Later, Gu et al. [63] studied and dissected the specific and overlapping roles of Rac1 and Rac2 in HSC/Ps. Deletion of both Rac1 and Rac2 led to massive egress of HSC/Ps into the PB from the BM. They also found that $Rac1^{-/-}$, but not $Rac2^{-/-}$, HSC/Ps failed to engraft in lethally irradiated recipient mice. The Rac 2 deficient HSC/Ps showed normal short-term engraftment, however, Rac2 null HSC/Ps showed reduced adhesion to fibronectin in vitro, which explained the impaired BM retention and increased mobilization seen in the $Rac2^{-/-}$ HSC/Ps. HSC/Ps lacking Rac2 showed increased apoptosis due to defective activation of the PI3K/Akt survival pathways. The Rac1 and Rac2 mutant phenotypes, such as defective proliferation, reduced adhesion to

fibronectin, impaired migration towards a SDF-1α gradient, and increased mobilization, were more severe in HSC/Ps lacking both Rac1 and Rac2, suggesting that Rac1 and Rac2 play overlapping roles. However, when analyzed carefully, it was found that deletion of *Rac1* after engraftment did not impair steady-state hematopoiesis [68]. The engraftment failure of $Rac1^{-/-}$ HSC/P was due to impaired spatial localization with respect to the BM endosteal niche. However, $Rac1^{-/-}$ HSC/Ps showed normal homing to the BM medullary cavity. Although $Rac2^{-/-}$ HSCs showed near normal short-term engraftment, they were impaired in their long-term hematopoietic reconstitution due to an abnormal interaction with the BM microenvironment [69]. Sanchez-Aguilera et al. [70] in a gene-targeted mouse model demonstrated that Vav1, an upstream activator of Rac GTPases, controls the retention of quiescent HSCs near $nestin^{+}$ mesenchymal stem cells. Transplanted $Vav1^{-/-}$ HSP/Cs showed impaired early localization near $nestin^{+}$ perivascular mesenchymal stem cells. The engraftment defect seen in the $Vav1^{-/-}$ HSP/Cs was due to impaired responses to SDF-1α, decreased circadian- and pharmacologically-induced mobilization in vivo, and dysregulated Rac/Cdc42 activation.

Cdc42 Controls HSC Polarity and Asymmetric Cell Division

Asymmetric cell division (ACD) is a process in which cytokinesis of a mother cell generates two daughter cells of unequal fate. This process is a critical regulator of embryonic development and adult tissue stem cell homeostasis [71, 72]. In most cases one of the daughter cells behaves as a mother cell and replenishes the stem cell pool, and the second acquires a matured phenotype for tissue regeneration and repair. The molecular and cellular mechanisms of ACD have been extensively studied in the development of invertebrate embryos [72, 73]. In higher mammals, ACD is well characterized in mature epithelial cells and neural stem/progenitor cells [74, 75]. The basic mechanisms and key regulators of ACD between invertebrate and higher mammalian cells are highly conserved [76, 77]. ACD is accomplished in three sequential steps; (1) establishment of the axis of polarity during late interphase or early prophase; (2) orientation of the mitotic spindles along the axis of polarity; and (3) cell division leading to the formation of two unequal daughter cells harboring different polarity proteins and cell fate determinants [71]. During the initial stages, the cell polarity proteins and cell fate determinants asymmetrically localize to the opposite poles of the cells, and thereby establish an axis of polarity [78]. The process of ACD begins with the polarized localization of apical polarity complex proteins PAR3 (Bazooka in *Drosophila*), PAR6 and atypical protein kinase C (aPKC) [79–82]. The apical polarity complex PAR3-PAR6-aPKC recruits other polarity regulators such as inscuteable (Ins) [79, 83], partner of inscuteable (Pin) [84, 85], and guanine nucleotide binding protein-αi (Gαi) [86] to the apical side of the cells. This is followed by basal recruitment of cell fate determinants such as Numb, Brat, Miranda, Prospero, and partner of Numb (PON) at the pro-metaphase stage of the cytokinesis [87–92]. The daughter cell harboring Numb, an endocytic

protein and Notch signaling inhibitor, is differentiated to the mature phenotype. Several studies support the hypothesis that aPKC dependent phosphorylation is critical for the asymmetric localization of Numb [71, 93, 94]. Following asymmetric distribution of polarity proteins and cell fate determinants, the next step is the correct orientation of the mitotic spindle along the axis of polarity. Pin recruits the microtubule motor proteins, dynein and kinesin, through its interaction with the dynein-binding protein Mud (Drosophila homologue of mammalian NuMA) and the Discs-large (Dlg)-kinesin heavy chain (Khc-73) protein complex, respectively, and thereby anchors the astral microtubule of the mitotic spindle to the apical cell cortex [95, 96].

The continuous rearrangement (assembly and disassembly) of actin and the microtubule cytoskeleton plays a decisive role in establishing the polarity of migrating fibroblasts, stem cells during embryonic development, neural stem/progenitor cells, and mature epithelial cells [1, 97]. The Rho family of small GTP-binding proteins, mainly Rac1, Rac2 and Cdc42, regulate the cell polarity by modulating the rearrangement of actin and the microtubule cytoskeleton through their interactions with actin and tubulin-binding protein [1]. The role of Rho GTPase Cdc42 in the establishment of the polarity axis during ACD is well documented in embryonic development and stem cell biology. Cdc42 activates the apical polarity complex PAR3-PAR6-aPKC by directly interacting with adaptor protein PAR6 through its Cdc42- and Rac-interactive binding (CRIB) domain and plays a critical role in establishing the axis of polarity in the Drosophila neuroblast, C. elegans zygote, and mammalian neural stem/progenitor cells [98, 99]. The interaction of Cdc42 and PAR6 activates the aPKC, a Ca^{++} and DAG (diacyl glycerol) independent serine/threonine kinase. The aPKC phosphorylates and directs the localization of the cell fate-determinant Numb toward the opposite pole of the cells [99, 100].

Unlike in invertebrate embryogenesis and mammalian neurogenesis, in HSCs, the mechanisms of ACD and the roles of polarity proteins and cell fate determinants are poorly understood. The development of the in vitro single cell culture and the paired daughter cells assay offered, for the first time, clues about the ACD of primitive HSCs [101, 102]. The daughter cells derived from one primitive ($Lineage^{-}c\text{-}Kit^{+}Sca1^{+}CD34^{-/lo}$) HSC possessed a differential ability for proliferation in vitro and hematopoietic reconstitution in vivo, suggesting the occurrence of ACD. Using the paired daughter cells assay, it was found that a majority of the primitive HSCs underwent ACD, whereas, the hematopoietic progenitors predominantly divided in a symmetric commitment manner. However, these studies did not provide direct evidence of the differential distribution of polarity proteins and subsequent ACD in HSCs. Using a Notch driven green fluorescent reporter system in a real-time microscopic imaging method, Wu et al. demonstrated that HSCs undergo both asymmetric and symmetric cell divisions [103]. In the case of ACD, the Notch inhibitor Numb was asymmetrically segregated into one of the daughter cells, and the daughter cell harboring Numb eventually differentiated and lost the Notch-driven expression of green fluorescent protein. Pro differentiation and pro self-renewal environments modulate the balance between the asymmetric and symmetric cell division of HSCs. Also, the presence of an oncogene has been shown to skew the

symmetry of cell division. Mushashi2 (Msi2), a cell polarity RNA binding protein, has been shown to regulate normal HSCs and leukemic stem cell activities [104, 105]. In neural progenitor cells, Msi1 directly binds Numb mRNA in the 3′ untranslated region, inhibits its translation and results in the down-regulation of the level of Numb protein, and the subsequent up-regulation of Notch activity [106]. In chronic myelogenous leukemia the progression of the disease from the chronic phase to the aggressive blast crisis phase is correlated with an increase in Msi2 and a decrease in Numb protein levels. An overexpression of Numb induces the differentiation and slows the disease progression [105, 107]. Together, these results suggest that ACD, and the expression and distribution pattern of the cell fate determinant Numb, regulates the activity of normal and malignant HSCs. However, contradictory reports exist about the roles of Numb, Numb-like protein, and the apical polarity complex PAR3-PAR6-aPKC, in the regulation of HSC activities. Wilson et al. [108] showed that the conditional and combined deletion of Numb and Numb-like protein did not affect the self-renewal or multi-lineage differentiation potential of HSCs. Also, the role of Msi2 has been further analyzed using a conditional gene-targeted mouse model [109]. Deletion of Msi2 resulted in depletion of quiescent HSCs due to increased differentiation, and the Msi2 loss-of-function phenotype is independent of Numb. In a conditional gene-targeted mouse model, Sengupta et al. [110] have shown that the constitutive or inducible deletion of the apical polarity complex proteins aPKC ζ and/or aPKC λ does not affect steady-state or stress-induced hematopoiesis. The percentage of polarized primitive HSC population remained unaffected in the absence of both homologues of aPKC. However, the role of these polarity proteins and cell-fate determinants in the regulation of malignant or leukemic stem cell cannot be ruled out.

Recently, the role of the Rho GTPase Cdc42 and the planar cell polarity proteins Frizzled (Fz) and Flamingo (Fmi) in the polarity and ACD of HSCs has been studied in detail. A summary of the role of Cdc42, and the core planar cell polarity proteins Fz and Fmi, in the regulation of ACD of HSCs is given in Fig. 3.3. In 2013, Florian et al. [111], first demonstrated the role of Cdc42 in the regulation of HSC polarity and ACD. In this study, the polarized distribution of tubulin and Cdc42 was used as a surrogate marker for the quantitation of HSC polarity. The level of GTP-bound activated Cdc42 critically regulates HSC polarity. Aged HSCs with elevated Cdc42 activity lost their polarity and long-term repopulation potential in vivo. Casin, a Cdc42 specific Rho GTPse inhibitor, induced HSC polarity and rejuvenated the aged HSCs, with an in vivo long-term reconstitution potential similar to young HSC. The pharmacological inhibition of Cdc42 activity in aged HSCs restored the level and spatial distribution of histone H4 lysine 16, suggesting a role for Cdc42 in the epigenetic regulation of HSCs. The canonical Wnt/β-catenin signaling pathway regulates the self-renewal of HSCs [112]. The non-canonical Wnt signaling pathway, mediated through the planar cell polarity proteins Fmi, Fz and disheveled (Dsh) has been shown to maintain the quiescent long-term primitive HSCs [113]. Fz, a seven-span transmembrane protein, and Fmi, a cadherin family transmembrane protein, are highly expressed and distributed in a polarized manner in primitive long-term HSC. Fz mediated non-canonical Wnt signaling suppresses the Ca^{2+}-NFAT-IFNg

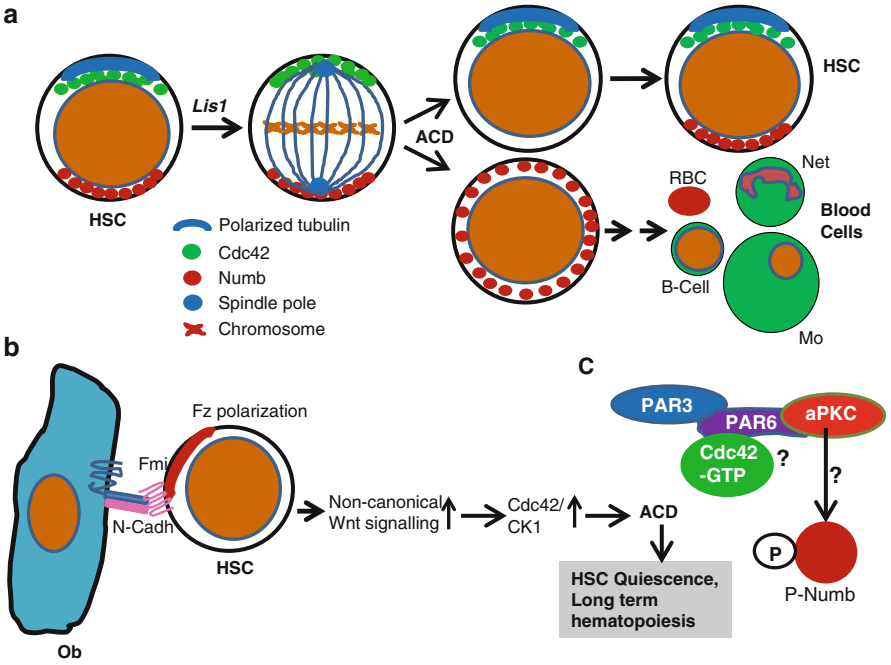

Fig. 3.3 Cdc42 regulates the polarity and asymmetric cell division of hematopoietic stem cells (HSCs). (**a**) The polarized nature of HSCs is determined by analyzing the asymmetric cell distribution (ACD) of Cdc42, Numb, and tubulin. The polarized distribution of Cdc42 and tubulin establishes the axis of polarity, with Numb and Cdc42 at the opposite poles. However, the cellular distribution of tubulin and Cdc42 with respect to Numb has yet to be analyzed in the same HSC. Lis1, a dynein binding protein, regulates the spindle orientation and ensures the occurrence of both symmetric and asymmetric division, and thereby, the self-renewal versus differentiation potential of HSCs. The committed progenitors generate mature red blood cells (RBC). (**b**) The core planar cell polarity (PCP) proteins, Flamingo (Fmi) and Frizzled (Fz), regulate HSC quiescence through the activation of non-canonical Wnt signaling in the endosteal niche. The interaction of Fmi, a cadherin-like transmembrane receptor expressed in the HSC, with N–cadherin (N-Cadh) expressed in the osteoblast (Ob) guides the polarized distribution of seven-span transmembrane receptor Fz at the Ob/ HSC interface, and activates non-canonical Wnt signaling mediated through the Cdc42/casein kinase-1 (CK1) pathway. The activated Cdc42 regulates HSC ACD and quiescence, and long term hematopoiesis. (**c**) The sub-cellular distribution of the apical polarity protein complex PAR3-PAR6-aPKC has not yet been analyzed in HSCs in detail. The interaction of GTP-bound Cdc42 with PAR6 and direct evidence of aPKC mediated Numb phosphorylation and asymmetric distribution of p-Numb is yet to be established in HSCs. *Mo* monocyte, *Net* neutrophil, *P* phosphorylation, *CK1* casein kinase-1

pathway through the Cdc42-PAK1-CK1 (casein kinase1) complex, and antagonizes the canonical Wnt signaling pathway. Another intriguing report describes how the switching from the canonical to non-canonical Wnt pathway takes place in aged HSCs [114]. In aged long-term HSCs a dramatic increase in the expression of Wnt5B was shown to activate the non-canonical Wnt signaling pathway, while

inhibiting the canonical Wnt/β-catenin pathway. Treatment of young HSCs with Wnt5B resulted in the activation of Cdc42 and the loss of HSC tubulin polarity, and with Wnt5B resulted in the aged HSC phenotype in vivo. These studies offer concrete evidence for the role of Cdc42 in the establishment of HSC polarity and subsequent ACD. However, it has yet to be demonstrated in HSC whether PAR6 is the direct target of activated Cdc42 for the activation of the apical polarity complex PAR3-PAR6-aPKC and the subsequent aPKC-mediated phosphorylation of Numb resulting in its asymmetric segregation to one of the daughter cells. It is also possible that activated Cdc42, through its interaction with the WASP/Arp2/3 complex, induces actin polymerization in a polarized manner, and that the crosstalk between actin and the microtubule cytoskeleton regulates the tubulin polarization of HSCs.

Two recent reports demonstrating the roles of the dynein binding protein Lis1, and the acto-myosin complex protein myosin II (MIIB), in the ACD of HSCs, further indicate that actin and the microtubule cytoskeleton regulate HSC polarity and asymmetric division. Lis1 facilitates the anchoring of the mitotic spindle to the cell cortex through its interaction with dynein and the dynactin complex, and ensures the proper orientation of the mitotic spindle during cell division [115]. The deletion of *Lis1* results in the depletion of HSC pools, a bloodless phenotype and embryonic lethality. The incorrect positioning of the mitotic spindle and a defective inheritance of the cell fate determinant Numb in the absence of Lis1 led to accelerated differentiation of HSCs and exhaustion of the stem cell pool. Recently, MIIB has been shown to be distributed in a polarized manner in HSCs and to regulate the ACD. The RhoA effector protein, Rho kinase (ROCK), induced the phosphorylation and activation of the myosin light-chain that in turn induced the acto-myosin contractile force required for the formation of actin stress fibers and focal adhesion contact. The regulation of ACD by MIIB tempted us to speculate on a direct role for RhoA GTPase in this process [116].

Rho GTPases and Human HSC/P Disease

The importance of Rho GTPase regulated signaling pathways in human biology is highlighted by the identification of genetic alterations in all classes of protein that interact with the GEFs, GAPs, GDIs, receptors, and downstream targets. Rho GEF genes rearrangements and deletions, have been identified in developmental and neuro-degenerative disorders, and in cancer.

Rho GTPases and Leukemia and Lymphoma

Many GEFs, including Dbl, Lbc, Lfc, Vav, Net1, Ect2, and Tiam, were originally isolated as oncogenes using an in vitro NIH 3T3 fibroblast transformation assay with DNA derived from various human tumors [25, 117–120]. Further studies revealed that constitutively active Rho, Rac, or Cdc42 also induced transformation,

strongly suggesting that the oncogenic activity of GEFs is mediated through dysregulated activation of Rho GTPases [121].

In lymphohematopoiesis, Rho GTPase activity has been implicated in the transformation of HSCs or hematopoietic progenitors acquiring self-renewal ability. For instance, the Rho family of GTPases has been implicated in the pathogenesis of leukemias and lymphomas.

BCR-ABL leukemias result from the reciprocal translocation between the BCR gene on Chromosome 22 and the Abelson leukemia (ABL) gene on Chromosome 9. Several types of fusion protein can be generated including the forms p210-BCR-ABL and p190-BCR-ABL. The wild-type BCR fragment contains a DH-PH GEF domain located centrally, and a GAP domain in its C-terminus. In BCR-ABL leukemias, the reciprocal, 9:22 chromosome translocation results in a fusion of the N-terminal sequences derived from BCR, with the non-receptor tyrosine kinase ABL. The two most common BCR-ABL fusion proteins are a 210 kDa protein, which contains the DH-PH domains, but not the GAP domain and is associated with chronic myeloid leukemia (CML), and a 190 kDa protein which lacks the DH-PH and GAP domains of BCR and is associated with acute lymphoblastic leukemia (ALL).

CML is a hematological malignancy that is characterized by an uncontrolled expansion of immature myeloid cells and their premature release into the circulation. CML is caused by the expression of the fusion oncoprotein p210-BCR-ABL, a constitutively active tyrosine kinase which regulates a variety of signaling cascades, including Ras, extracellular-signal regulated kinase (ERK), Akt, c-Jun activated kinase (JNK), p38, CrkL, signal transducer and activator of transcription 5 (STAT5), and nuclear factor-κB (NF-κB) [122]. Expression of p210-BCR-ABL, confers a proliferative advantage to cells, and induces abnormal adhesion and migration of hematopoietic progenitor cells [123, 124]. These effects can be suppressed by the tyrosine kinase inhibitor, imatinib mesylate. Imatinib is a highly effective drug in CML treatment, however, tyrosine kinase inhibition resistance due to mutations in p210-BCR-ABL and other causes of HSC resistance to drug treatment have increased the interest in better defining the signaling pathways activated by p210-BCR-ABL [125].

p210-BCR-ABL contains additional functional domains of interest. In particular a DH domain with GEF that can activate Rho GTPases, and a Src-homology3 (SH3) domain which can recruit other proteins with GEF activity as well as Rac proteins, which have been shown to activate a variety of signaling molecules that coincide with known downstream targets of p210-BCR-ABL. Hyperactivation of Rac1 and Rac2 and, to a lesser extent, Rac3 in HSC/P isolated from chronic phase CML patients has been demonstrated [126]. In an experimental model of CML in mice, Rac GTPases were also shown to be hyperactivated in primary HSC/P expressing p210-BCR-ABL after retrovirus-mediated gene transfer [126]. While a Rac1 deficiency did not modify the median survival of p210-BCR-ABL-expressing mice, the median survival of p210-BCR-ABL-expressing Rac2-deficient mice was significantly increased (from 21 to 43 days), and the median survival of p210-BCR-ABL-

expressing Rac1/Rac2-deficient mice was even more strikingly increased (to 92 days). Expression of p210-BCR-ABL in Rac1/Rac2-deficient HSC/Ps also led to an altered disease phenotype, with mice showing oligoclonal leukemias of myeloid, lymphoid, or bi-lineage immunophenotypes. In this murine model, ERK, JNK, p38, Akt, STAT5, and CrkL signaling were all attenuated in splenocytes harvested from p210-BCR-ABL-expressing Rac2-deficient leukemic recipients, and almost completely abrogated in the Rac1/Rac2-deficient cells. Mechanistic studies in a binary transgenic animal model of inducible CML-like disease further unveiled the specific role of Rac2. These studies demonstrated that Rac2 is required for leukemogenesis and is a potent therapeutic target for CML expressing p210-BCR-ABL, where it is required for the proliferation and survival of leukemic stem cells and progenitors [127].

These alterations in signaling correlated with the overall survival seen in animals from each of these genotypes. The decreased activation of downstream pathways was not due to decreased ABL tyrosine kinase activity, as autophosphorylation of p210-BCR-ABL was still noted in these cells [126]. STAT5 phosphorylation was also detectable in leukemic cells regardless of the presence or absence of Rac1 and Rac2 GTPase activity. Activation of CrkL, which has been suggested as an effector that binds directly to p210-BCR-ABL [128], was decreased in Rac2-deficient and practically abrogated in Rac1/Rac2-deficient leukemias, suggesting that CrkL activation is dependent on other proteins as well. These data suggested that Rac1 and especially Rac2 were critical for p210-BCR-ABL transformation and myeloproliferative disorder (MPD) development in vivo. Interestingly, Rac3 appeared hyperactivated in splenocytes derived after long latency in Rac1/Rac2-deficient animals. Rac3 was originally discovered by screening the p210-BCR-ABL-expressing erythroid blastic-phase CML cell line K562 [129]. Rac3 activation has been demonstrated in p190-BCR-ABL-expressing malignant precursor B-lineage lymphoblasts [130] suggesting that Rac3 hyperactivation could play a specific role in cancer development and invasiveness. These data, along with the observed differences in survival mediated by Rac1- versus Rac2-deficient HSCs, support the hypothesis that individual Rac GTPases play unique roles in the development of p210-BCR-ABL-mediated disease.

Based on these genetic data, the effect of the Rac inhibitor NSC23766 on p210-BCR-ABL induced transformation was examined. NSC23766, a first-generation, Rac-specific small molecule inhibitor [131], was developed using computer-assisted virtual screening based on the GEF-Rac1 GTPase complex. NSC23766 was found to fit into a shallow surface groove of Rac1 that has been shown to be critical for GEF-specification. It was shown to effectively inhibit Rac protein binding and activation by the Rac-specific GEFs TrioN or Tiam1 in a dose-dependent manner. In contrast, NSC23766 did not interfere with the binding or activation of Cdc42 or RhoA by their respective GEFs. NSC23766 induced decreased Rac-dependent p21-activated kinase (PAK) activation and mobilization of normal HSCs in mouse studies [132]. In either the murine model of p210-BCR-ABL-induced MPD or in a human xenogeneic transplantation assay, NSC23766 inhibited Rac GTPase activity

and impaired leukemogenesis [131]. These studies defined Rac as an attractive molecular target in p210-BCR-ABL transformed HSCs.

Interestingly, p190-BCR-ABL which lacks a GEF-domain, also induces activation of the Rho family of GTPases, specifically Rac and Rho, and induces pre-B lymphoblastic leukemias which are initiated by the transformation of B-cell progenitors. p190-BCR-ABL recruits other GEFs to activate the Rho family of GTPases. Vav3, but not its homologues Vav1 or Vav2, was found to be required for p190-BCR-ABL lymphoblastic leukemogenesis, proliferation, and especially leukemic progenitor survival. Forced expression of Vav3 restored leukemogenesis, and a deficiency of Rac2 phenocopied the effect on leukemogenesis impairment which had been induced by the Vav3 deficiency. Vav3 mediated activation of Rac and Rho seems to repress the expression and/or activation of pro-apoptotic BH3-only molecules, included Bax, Bak, Bad, Bim or Bik [133].

The mixed-lineage leukemia gene (MLL) on chromosome 11q23 is rearranged in both acute myelogenous leukemia (AML) and acute lymphoblastic leukemias (ALL) and constitutes a group of leukemias associated with poor prognosis. These MLL gene rearrangements consist of reciprocal translocations that fuse the amino terminus of *MLL* to a diverse group of partner genes [134, 135]. In a fashion similar to p210-BCR-ABL CML, the leukemia initiating cell is a HSC or progenitor with HSC-like characteristics [136], and MLL rearrangements can be found in up to 7–10 % of acute leukemias. In AML, the most common partner gene for *MLL* is *AF9* on chromosome 9p22. Recently, Somervaille and Cleary [137] showed that the activity of the small Rho GTPase proteins Rac1 and Cdc42 are increased in murine cells expressing MLL-AF9. In a xenograft model of MLL-AF9 leukemia, Wei et al. [138] targeted the Rac1 signaling pathway pharmacologically or by gene-silencing, which resulted in rapid apoptosis of *MLL-AF9*-expressing cells. Confirming these data, in a panel of AML cell lines, Muller et al. demonstrated that the MLL gene-rearranged cell lines ML-2 and THP-1 displayed a profound dependence on Rac signaling, and treatment with NSC23766 inhibited the growth of these cells in vitro, and in an xenograft model in vivo [139].

T-cell lymphomas are a heterogeneous and poorly understood group of non-Hodgkin lymphomas. Angioimmunoblastic T-cell lymphomas (AITL) are characterized by skin rash, generalized lymphadenopathy, splenomegaly, pleural effusion, ascites, anemia and thrombocytopenia, and an increase of circulating large granular lymphocytes with CD3(−) and CD16(+), CD56(+) with T-cell receptor γ-chain gene rearrangement. Although these lymphomas seem to arise from more differentiated T-cells, the cellular origin of these lymphomas is unclear and a cellular origin in a T-cell progenitor/precursor cannot be ruled out. In AITL, highly prevalent RHOA mutations encoding a p.Gly17Val alteration are present in approximately two-thirds of cases. RHOA Gly17Val protein seems to interfere with RHOA signaling in the downstream activation of Rho effectors in biochemical and cellular assays, an effect potentially mediated by the sequestration of activated GEF proteins [140, 141]. The mechanism behind how this loss-of-function mutation induces lymphomagenesis is unknown.

Rho GTPase Activity in HSC Activity in Vasculopathies and Sickle Cell Disease

Unlike their intrinsic activity in leukemias and lymphomas, Rho GTPase activity has also been recognized as a major feature of complications in non-malignant disease. Patients in organ failure of vasculo-endothelial origin have an increased circulating pool of HSC/P [142, 143] which may represent a homeostatic stress response that contributes to vascular damage repair [144]. An example of systemic vasculo-endothelial disease is sickle cell disease (SCD). SCD results from the substitution of a single nucleotide, valine to glutamic acid, at the sixth amino acid of the β-globin chain of hemoglobin A. SCD is characterized by globin polymerization that results in red cell dehydration, hemolysis and subsequent stress erythropoiesis. The common features of SCD are the activation of multiple signaling pathways associated with endothelial damage [142], and an increased pool of primitive hematopoietic progenitors is found in circulation [143]. Vascular pathology is a common feature of SCD patients [145]. Recent data have associated hyperangiotensinemia in SCD with increased circulation of HSC/P, including primitive progenitors at an unmatched magnitude with increased levels of circulating erythroid committed progenitors. The mechanism postulated is that hyperangiotensinemia results in HSC/P de-adhesion from BM endothelial cells through changes in the balance of activated Rho family GTPases, Rho and Rac, and cytoskeletal rearrangements in BM endothelial cells (BMEC) and HSC/P [146].

Concluding Remarks

After more than 20 years of elucidation of the crucial roles of the Rho family of GTPases in the regulation of basic cytoskeletal activities, we are still unveiling some of their functions. Changes in activity through induction, repression, activation, or inhibition, through a complex array of signaling pathways control the migration, adhesion and transformation of HSC/P. This chapter is far from complete. It is expected that in the near future, more information will become available about the multiple mechanisms regulated by the Rho family of GTPases which will be crucial to our understanding of the basic mechanisms of cell division, migration, and the transformation of hematopoietic cells, and enable us to develop novel targeted therapies in hematopoietic disease.

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