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Hematopoietic-specific Rho GTPases Rac2 and RhoH and human blood disorders

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Abstract

The small guanosine triphosphotases (GTPases) Rho proteins are members of the Ras-like superfamily. Similar to Ras, most Rho GTPases cycle between active GTP-bound, and inactive GDP-bound conformations and act as molecular switches that control multiple cellular functions. While most Rho GTPases are expressed widely, the expression of Rac2 and RhoH are restricted to hematopoietic cells. RhoH is an atypical GTPase that lacks GTPase activity and remains in the active conformation. The generation of mouse knock-out lines has led to new understanding of the functions of both of these proteins in blood cells. The phenotype of these mice also led to the identification of mutations in human *RAC2* and *RHOH* genes and the role of these proteins in immunodeficiency diseases. This review outlines the basic biology of Rho GTPases, focusing on Rac and RhoH and summarizes human diseases associated with mutations of these genes.

Keywords

Rho GTPases; Rac; RhoH; cytoskeleton; hematopoiesis

The Rho family of GTPases

Rho GTPases are highly conserved low molecular weight proteins that integrate receptor-mediated signals and regulate vital cellular functions via differential binding to effectors and regulators in the active versus inactive conformation. Ha-Ras, the first member of the small GTPase family, was identified in 1981 and is considered as founder of the family[1]. Meanwhile the entire family comprises more than 150 members organized into 6 main families: Ras, Rho, Rab, Arf, Ran and MIRO [2]. Most Rho GTPases act as molecular

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switches to control different aspects of cytoskeleton rearrangement, trafficking to cellular compartments, cell cycle regulation, gene transcription, cell growth and some have also been implicated in malignant cell transformation [3,4]. The characteristic feature of this family is their conserved structural backbone that consists of five G-boxes that are involved in GDP binding and exhibit GTPase activity[5]. In the GDP-bound state these molecules are inactive, while after binding of GTP they obtain an active conformation and are then able to interact with different downstream effectors mediating diverse cellular effects (Figure 1A shows Rac GTPase)[6]. These conformational changes are influenced by guanine nucleotide exchange factors (GEF) that catalyze replacement of GDP by GTP and are thus activating, GTPase activating proteins (GAP) that accelerate the intrinsic GTPase activity and therefore negatively regulate Rho activity and guanine nucleotide dissociation inhibitors (GDI) that also stabilize the inactive, GDP-bound form in the cytoplasm[7,8]. Like Ras proteins, Rho GTPases also undergo posttranslational modifications such as lipid prenylation at the conserved carboxy-terminal sequence that together with the polybasic domain regulates their subcellular localization to the membrane and critically influences protein-protein-interactions[9]. Due to their participation in cytoskeletal reorganization Rho GTPases control vital cellular processes like cell shape, adhesion and migration. Therefore they are required for proper trafficking, homing and engraftment - all processes that are not only relevant in hematopoietic stem cells but also key features of immune cells like lymphocytes and neutrophils[10,11]. Whereas most members of this protein family are ubiquitously expressed some of the subfamilies exhibit tissue-specific expression suggesting that these proteins are critical for more specialized regulatory functions. Amongst these, Rac2 and RhoH have been demonstrated to be specifically expressed in hematopoietic cells[12,13,14]. Interestingly, alterations in both proteins have recently also been implicated in human diseases of immune function.

Rac GTPases

The Rac subfamily of GTPases encompasses three highly homologous members Rac1, Rac2 and Rac3. Rac2 shows hematopoietic-specific expression while Rac1 and Rac3 are widely expressed. Besides RhoA and Cdc42, Rac is one of the best studied Rho GTPase subfamilies. Initial studies in fibroblasts revealed the involvement of these molecules in regulation of actin cytoskeleton changes[15]. Whereas RhoA induces stress fiber formation and mediates focal adhesion, Cdc42 is required for filopodia and microspike formation whereas Rac has been implicated in lamellopodial extension and membrane ruffling[16]. Besides the above mentioned functions of Rac proteins in cellular migration and activation of the p42/44 and p38 MAPK, JNK and Akt pathways in various cellular systems, Rac2 has been identified as a component of the phagocytic oxidase complex in neutrophils (Figure 1A) and its deficiency results in impairment of both neutrophil chemotaxis in response to Formylmethionyl-Leucyl-Phenylalanine (fMLP) and superoxide production and is therefore associated with phagocyte immunodeficiency in humans[17,18,19,20,21,22,23]. Rac2 differs from Rac1 and Rac3 with regard to the primary “polybasic domain” located near the c-terminal CaaL conserved sequence mediating lipid modification, which is analogous to the Ras “hypervariable region” and critical for intracellular protein localization[24,25].

RhoH

While Rac GTPases retain the characteristic ability of Rho GTPases to cycle between an inactive GDP-bound and active GTP-bound state, RhoH is an atypical Rho GTPase which is GTPase deficient and therefore remains in a constitutively active, GTP-bound state[26,27]. This implies that RhoH activity depends on its expression level and posttranslational modifications. We have previously demonstrated that RhoH activity is modified after TCR engagement by phosphorylation of its unique ITAM-like motif[28]. *RHOH* was first identified as a fusion partner and hypermutable gene in malignant lymphoma and high expression levels of RhoH have been detected in lymphocytes[29,30,31]. This predicts the disease phenotype observed both in *Rhoh* knock-out mice and humans with mutations of *RHOH*. RhoH deficiency appears to mostly affect T cell development and function due to its requirement for TCR mediated activation of ZAP70, LCK and the LAT signalosome regulating thymocyte proliferation, survival and selection (Figure 1B and Figure 1C)[28,32,33]. Thus, lack of RhoH results in T cell deficiency and impaired T cell function both in humans and mice[34,28].

Cross-talk between Rho GTPases

Interestingly, other Rho GTPase family members such as RhoE have been shown to mediate opposing effects compared to RhoA such as disassembly of stress fibers and inhibition of ERK activation. These data suggest the presence of crosstalk between GTPases (Figure 2A)[35,36]. Supporting this general theme, whereas Rac and Cdc42 have been shown to activate the c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathway and mediate nuclear signaling via NFkB, RhoB and RhoH inhibit these signaling pathways in various cell systems[27]. We have recently demonstrated that lack of RhoH expression also influences Rac and RhoA activity as well as their membrane localization in hematopoietic stem / progenitor cells (HSC / P) and leukemia cells (Figure 2B)[37,38]. Thus, RhoB, RhoD, RhoE and RhoH appear to have antagonistic effects on cellular functions compared to Rac, RhoA and CDC42 indicating that Rho GTPases act within an intricate network competitively influencing downstream target molecules or even interacting with each other or associated regulatory molecules in an agonist and cell-specific fashion[35,39,27,37].

Rho GTPases in human disease

A limited number of small GTPases are implicated in human diseases. *RAS* and, as mentioned above, *RHOH* mutations have mainly been associated with malignant transformation and tumor progression in humans[40]. RhoH harbors amino acid substitutions at codons 12, 13, 59 and 61 (based on Ras amino acid sequence numbering), which are analogous to mutations in oncogenic Ras proteins, leading to a constitutive GTP-bound, active protein[14,41,27,42]. Unlike in oncogenic Ras, mutations of coding sequences of *RHO GTPases* have rarely been reported in human cancers, while alterations in protein levels have been demonstrated for several Rho GTPases in solid tumors[43,44,45] and leukemic cells[46,47]. Recently an activating mutation *of RAC1 (P29S)* within the highly conserved switch I domain of the molecule has been identified as a frequent alteration in melanoma promoting melanocyte proliferation and migration[48 49]. *RHOH* mutations found in B cell

lymphoma affect non-coding, presumably regulatory regions suggesting that RhoH protein levels may be critical for the survival of malignant B cells[50,51,52].

Mutations within the coding region of *RAC2* and more recently *RHOH* have been identified in patients presenting with disorders restricted to the immune system[53,23,54,34]. Moreover, mutations affecting the WASP pathway result in Wiscott Aldrich Syndrome, a well characterized X-chromosomal inherited disease entity that is characterized by eczema, thrombocytopenia and immune deficiency[55]. We will focus on Rac and RhoH GTPases in this review.

To study the physiologic consequences of Rac2 and RhoH deficiency in more detail *in vivo*, murine knock-out models have been created by homologous recombination resulting in disruption of the respective gene locus[56,57,58,28]. This genetic approach has been particularly informative in the case of Rac2, due to the high degree of sequence homology between the three Rac isoforms, which previously led to difficulty in assigning functions to each individual protein.

The Rac2 knock-out mouse model

The use of constitutively active (CA) and dominant negative (DN) mutants as well as expression of knock-down vectors *in vitro* suggest that the different Rac isoforms most likely share common but also redundant functions[59,22]. Several years ago, due to its restricted expression pattern, we hypothesized that Rac2 may have distinct and unique functions that were agonist and cell type specific. Overall, the genetic knock-out of *Rac2* and later the conditional knock-out of *Rac1* allowed us and others to demonstrate that in phagocytic cells Rac1 and Rac2 are differentially required for both chemotaxis and generation of phagocytic oxidants needed for bacterial killing[56,60,24]. The differences of a few amino acids in c-terminal sequences of Rac do influence the strength of interaction of the GTPase with p67phox within the oxidase complex and regulates intracellular localization of the activated GTPase and therefore mediate distinct cellular functions. *Rac2−/−* mice develop normally and are fertile[56]. However, despite normal bone marrow cellularity, Rac2 GTPase function is required for HSC and P adhesion and mobilization both *in vitro* and *in vivo*. *Rac2−/−* HSC/P cells exhibit a cell-intrinsic defect in microenvironment interaction that leads to defective long-term engraftment in competitive and secondary transplantation assays[61]. Nonetheless, hemoglobin and T cell numbers are normal in peripheral blood of *Rac2−/−* mice. Interestingly, T cell numbers are markedly reduced in *Rac1* and *2* double knockout animals, indicating that Rac1 and Rac2 may have redundant roles in TCR signaling and during T cell development[62]. Mast cells require Rac2 for migration, degranulation and integrin-mediated adhesion as well as growth-dependent survival mediated by Akt activation[63].

Rac2−/− mice demonstrate prominent leukocytosis due to a massive increase in mature neutrophils detectable in their peripheral blood. As this could be the consequence of defective neutrophil migration and chemotaxis, functional studies investigating cytoskeletal changes, adhesion and migration of Rac2 deficient neutrophils were undertaken. These studies demonstrate that the phenotype is caused by abnormal F-actin assembly, reduced

tethering to GlyCAM-1 and defective chemotaxis in response to fMLP, IL8 and LTB4. These cellular alterations most likely contribute to leukocyte trapping within the circulating blood. In addition, these mice have an increased susceptibility to invasive fungal infections, likely contributed to by reduced FcR mediated phagocytosis, reduced NADPH oxidase production in response to fMLP and reduced azurophilic granule release in the absence of Rac2 (Figure 1A)[56]. The fact that Rac1 is also mislocalized in the absence of Rac2, may further explain the severe defects in superoxide generation and migration observed in *Rac2−/−* neutrophils. Interestingly, migration and oxidase production in response to fMLP are normal in *Rac1−/−* neutrophils, while Rac1 and 2 deficient neutrophils are characterized by an even more pronounced phenotype, supporting the notion that Rac1 and 2 display both redundant and unique roles in neutrophils[64,65].

Observations made following overexpression of a DN D57N Rac2 mutant further support potential overlapping roles of Rac1 and Rac2 in neutrophils. Exchange of aspartic acid to asparagine at position 57 weakens GTP-binding of Rac2 while binding to GDP remains unchanged[23]. In addition, the mutated molecule is unresponsive to activation by GEFs such as TrioN and likely sequestering endogenous GEFs from other GTPases thus acting in a dominant negative fashion. Similar to deficiency of Rac2, expression of the D57N mutant in *Rac2−/−* and wild type (WT) BM cells is associated with compensatory increased endogenous protein expression of Rac1 that is less activated, while it has no impact on Cdc42 expression[23]. Accordingly, directed migration, engraftment and hematopoietic reconstitution *in vivo* is impaired in D57N Rac2 transduced *Rac2−/−* and WT HSC and D57N-expressing Rac2 neutrophils show a severe defect in superoxide production and chemotaxis. This phenotype is of particular interest since this D57N mutant (G169A *RAC2* gene mutation) has been identified in two patients suffering from severe phagocytic immunodeficiency[23] (see below).

Rac2 mutations cause severe myeloid dysfunction in humans

Neutrophils are the most abundant phagocytic cell type present in the peripheral blood. After neutrophils receive the appropriate signals, these cells quickly migrate to the site of infections and exert anti-microbial activity by phagocytosis, release of bactericidal substances or generation of neutrophil extracellular traps. Therefore these cells are a vital component of the innate immune system and granulocyte dysfunction increases susceptibility to acquired and recurrent, life-threatening infections.

Based on the described phenotype of *Rac2−/−* mice, two patients have been identified in whom the above mentioned dominant negative D57N mutation affecting the GTP-binding pocket of the *RAC2* gene occurred spontaneously and lead to severe phagocytic immunodeficiency in both children[53,66,23,54]. Whereas the first patient reported in 2000 presented with clinical symptoms that closely resembled the phenotype observed in *Rac2−/−* mice, the second patient was ascertained by abnormal T cell receptor excision circle (TRECs) patterns in universal newborn screening for congenital immunodeficiency disease in Wisconsin in 2009.

Both neonates had in common life-threatening bacterial infections including omphalitis during early infancy that required antibiotic treatment and surgical intervention. In the course of time, the first patient also developed an infected uracheal cyst and multiple perirectal abscesses, while the second child came to attention with a bacterial paratracheal abscess. Interestingly, in both patients leukocytosis with a significant neutrophilia was noted in the peripheral blood, while debridement revealed absence of pus at the sites of infection and biopsies showed sparse infiltrating neutrophils. Neutrophils displayed defects in fMLP-induced F-actin formation, chemotaxis and migration. In keeping with the murine phenotype, the first described patient's neutrophils also evidenced selective defects in superoxide production *in vitro*. Leukocyte adhesion deficiency type I was ruled out by normal CD11b/CD18 expression in flow cytometry. Surprisingly, in contrast to classical severe combined immunodeficiency (SCID) found by newborn screening, patient 2 had only mild T cell lymphopenia with a modest increase in T cells that displayed an effector memory phenotype in this newborn[54]. Normally low TRECs numbers result from decreased output of naïve T cells from the thymus and dilution of these cells by homeostatic proliferation. While deletion of both *Rac1* and *Rac2* in murine HSC inhibits production of common lymphocyte progenitors and suppresses T cell development in the thymus and peripheral organs, *Rac2* deletion alone causes defective receptor clustering during TCR stimulation and Th1 differentiation[62]. Thus, these data suggest a DN effect *in vivo* on Rac1 and Rac2 in human disease, a finding supported by the experimental data reported[23]. Alternatively, Rac isoform functions and their cross-compensation may well differ depending on the cell type and species.

The RhoH knock-out mouse model

RHOH mutations were initially implicated in the development of B cell malignancies[29,30,31]. Mutations have been found in the 5′ non-coding region and 1st intron of the gene, suggesting an effect on the expression level of the protein[14,67,68,31]. This is relevant since as noted above RhoH belongs to a group of atypical GTPases that have sequences analogous to transforming *RAS* mutations, lacking intrinsic GTPase activity and therefore remaining in a constitutively active, GTP-bound conformation[27]. Thus, it has been proposed that RhoH function is likely influenced by its expression level, mRNA stability and posttranslational modifications. RhoH expression is restricted to hematopoietic cells and high expression levels have been detected in the murine thymus and human T cells. Overexpression studies point to an inhibitory function of RhoH that suppresses the activity of other GTPases[58,27]. In Jurkat T cells it has been shown that RhoH can modulate integrin-mediated adhesion and regulate SDF1-induced T cell migration[69,70]. Increased RhoH expression levels in HSC are associated with impaired activation of Rac GTPases, reduced proliferation, increased apoptosis and defective actin polymerization and chemotaxis and result in defective hematopoietic reconstitution in transplantation assays[58]. Conversely, suppression of RhoH using RNA interference stimulates proliferation, survival and SDF-1 induced migration in HSC[58]. This antagonistic role of Rac and RhoH in cortical F-actin assembly and chemotaxis is mainly mediated by suppression of Rac membrane targeting and activation in primary HSC (Figure 2B)[58,37]. Li et al. have observed that RhoH also inhibits Rac mediated activation of p38 MAPK and NFkB signaling in Jurkat cells[27]. More recently it has been

postulated that RhoH determines sensing of chemokine-mediated “go” signals and TCR-dependent “stop” signals in T cells, thus regulating recirculation and APC interaction[71].

To study the effect of RhoH deficiency on development and function of hematopoietic cells *in vivo* and *in vitro* in more detail, *Rhoh*−/− mice have been developed by homologous recombination with the aim to disrupt the coding region of the gene[28]. *Rhoh*−/− mice develop normally and are fertile. These mice also have no obvious defect in HSC maintenance with normal BM cellularity. RhoH also appears not to be essential for erythroid, myeloid and B cell differentiation[33,28]. Nonetheless, RhoH is required for FcεRI-dependent signal transduction in mast cells and thus passive systemic anaphylaxis and histamine release are impaired in the absence of RhoH[72].

Moreover, *Rhoh*−/− mice are characterized by a severe defect in T cell development and demonstrate T cell lymphopenia in the blood, spleen and lymph nodes and reduced thymic cellularity[33,28]. The lack of mature, single positive (SP) peripheral T cells is caused by a block in T cell maturation at the double negative (DN) 3 and CD4 / CD8 double positive (DP) stage. The most immature DN cells can be further subdivided depending on their CD25 and CD44 expression. In the absence of RhoH, DN2 and DN3 cells are increased while CD25-CD44- DN4 are significantly decreased pointing to a block in thymic differentiation. During transition from a DN to DP stage TCRα- and TCRβ-expression and rearrangements are critical. While expression of TCRβ and CD3ε are normal in the absence of RhoH, surface expression of CD5 and CD69 is lower, indicating perturbation of positive selection. This suggests that RhoH is required for TCR-mediated signaling regulating positive selection of DP cells within the thymus. Despite normal TCR expression on *Rhoh*−/− T cells, these cells show decreased proliferation after CD3 stimulation, further suggesting RhoH involvement in TCR signaling. Indeed, *in vitro* binding assays and subsequent immunoprecipitation experiments identified Zap70 as a RhoH-interacting partner. Zap70 is a Syk family protein tyrosine kinase that is a vital component of the TCR signaling pathway (Figure 1B)[33,28]. Interaction with Zap70 SH2 domains is further increased in the presence of CA Lck after tyrosine phosphorylation of RhoH[32]. Non-phosphorylated RhoH ITAM mutants only partially rescue the T cell defect in *Rhoh*−/− mice indicating that the ITAM-like motif is functional and important for Zap70 interaction[28]. Moreover, recruitment of Zap70 to the plasma membrane and immunological synapse is abolished in agonist-stimulated *Rhoh*−/− T cells (Figure 1C) indicating that RhoH is a crucial regulator of the CD3ξ-Zap70 signaling pathway. In addition it has been demonstrated that RhoH interacts with inactive, c-src tyrosine kinase (CSK) phosphorylated Lck that is also involved in pre-TCR and TCR signaling[73]. The fact that TCR signaling is critically influenced by RhoH is further supported by the observation that in the absence of RhoH allogeneic kidney transplant rejection is reduced *in vivo* and alloreactivity is weaker *in vitro*[74]. Recent reports suggest a requirement of RhoH in ”agonist selection” of T cells where self-reactive T cells escape deletion and instead differentiate into natural regulatory T cells, NKT cells or TCRαβ CD8αα intraepithelial lymphocytes (IELs). While RhoH appears dispensable for the generation of TCRαβ CD8αα IELs, a subgroup of regulatory T cells mediating mucosal tolerance, generation of NKT cells and regulatory T cells is reduced in the absence of RhoH[75]. Taken together these data denote an important role of RhoH in TCR signaling via

recruitment of Zap70 and Lck to the immunological synapse and defines its role as an adaptor molecule in TCR function and T cell development in mice.

RhoH mutations in human disease

T cell development is critical for development of the adaptive immune system. After migration of common lymphoid progenitors from the bone marrow to the thymus, T cell maturation proceeds and involves TCR rearrangement, positive and negative selection as well as subsequent T cell proliferation and immune response mediated by mature T cells. Defects in T cell function have been implicated in numerous primary immunodeficiency diseases (PID) in humans.

We have recently described a consanguineous family with two siblings suffering from a rare genodermatosis, called *Epidermodysplasia verruciformis* (EV)[34]. The family was investigated for an underlying genetic predisposition applying genome wide linkage analysis by homozygosity mapping using a high-density SNP array and subsequent whole-exome sequencing of the putative disease-associated genes. Using this approach a homozygous nucleotide substitution at position 114 in exon 3 of the *RHOH* gene (*Y38X RHOH*) was identified in the affected siblings, while healthy family members were heterozygous for this mutation[34]. Despite normal mRNA expression levels, Y38X RhoH protein expression was severely diminished and in heterologous cells exhibited loss of function. EV is characterized by increased susceptibility to a specific group of weakly virulent keratinocyte-tropic human beta-papillomavirus genotypes and up to this point was not considered a PID[76]. Given the phenotype of *Rhoh*−/− mice, a more severe immunodeficiency might have been expected in humans.

Both affected individuals presented with persistent EV-HPV infections during early childhood that were accompanied by bronchopulmonary disease. Interestingly, given the previously identified relationship between *RHOH* mutations and B cell malignancies[34], childhood Burkett lymphoma was reported in one of these individuals[34]. Immunophenotyping revealed no major abnormalities in B cell subsets, NK cells, NKT cells, monocytes and polymorphonuclear cells. In addition, antibody levels were not reduced in these individuals[34]. Total T cell numbers were within normal range. However, CD4+ T cells were slightly decreased and CD8+ T cells increased in both affected individuals. Further detailed analysis revealed that both patients lacked naïve T cells and recent thymic emigrants, while effector memory and revertant memory T cell compartments exhibited signs of exhaustion. Restricted TCR usage and clonal expansion of certain TCR Vαβ was noted[34]. Similar to murine *Rhoh*−/− T cells, immortalized patient Samiri T cells failed to proliferate specifically in response to TCR stimulation and these cells lacked Zap70 phosphorylation, suggesting that RhoH is also a critical regulator of TCR signaling in human cells. This TCR signaling defect may contribute but likely does not sufficiently explain the localized susceptibility to EV-HPV observed in the patients. However, tissue-specific homing markers on circulating T cells, particularly β7+ T cells were reduced both in patients and *Rhoh*−/− mice, suggesting alterations in skin-homing T cell subsets, which could well confer weakness to the local immune barrier of the skin.

These findings indicate that RhoH deficiency leads to T cell defects and increases susceptibility to certain viral infections. The phenotype in humans may relate both to qualitative defects in TCR signaling and quantitative defects in specific skin-homing T cells. The relatively mild human phenotype compared to the murine knock-out is noteworthy and the potential role of RhoH in B cell malignancy remains largely unexplored.

Concluding remarks

Rho GTPases regulate a broad variety of vital cellular functions such as cytoskeletal rearrangements involved in cell shape, adhesion, migration, signal transduction, cell cycle progression and gene expression[3,4]. Of this large family of proteins, Rac2 and RhoH exhibit restricted expression in hematopoietic cells[12,13,14]. Rac family members have been implicated in lamellipodia formation, directed migration, chemotaxis and superoxide production in phagocytic cells[16]. Generation of a *Rac2*-deficient mouse line helped to determine the broader hematopoietic phenotype and revealed the presence of a phagocytic immunodeficiency that was specifically associated with lack of Rac2[22,23,59]. This was instrumental in identifying a DN *RAC2* mutation in a patient presenting with recurrent infections and leukocytosis closely resembling a neutrophil disorder previously observed in *Rac2*−/− mice and the human disease Leukocyte Adhesion Deficiency (LAD)[53,66]. Interestingly a second patient who had also spontaneously acquired the same *RAC2* mutation was ascertained by SCID newborn screening due to abnormal T cell parameters, but otherwise had a very similar phenotype[54].

Similarly, although *RHOH* has been initially identified in B cell lymphomas[14,67,68,31], generation of *Rhoh*−/− mice then revealed its requirement for normal T cell development and TCR signaling[28,33]. Meanwhile two children carrying a homozygous *RHOH* mutations resulting in a premature stop codon and non-functional RhoH protein have been identified[34]. In keeping with the findings in the murine *Rhoh*−/− model, these patients were characterized by a T cell deficiency that was associated with a specific, and apparently very focal, defect in the immune system and the rare skin disease EV. These examples highlight nicely the importance of genetargeted animal knock-out models to study the effect of specific gene functions *in vitro* and *in vivo* and emphasize that such murine models may help predict unexpected and previously molecularly uncharacterized human phenotypes.

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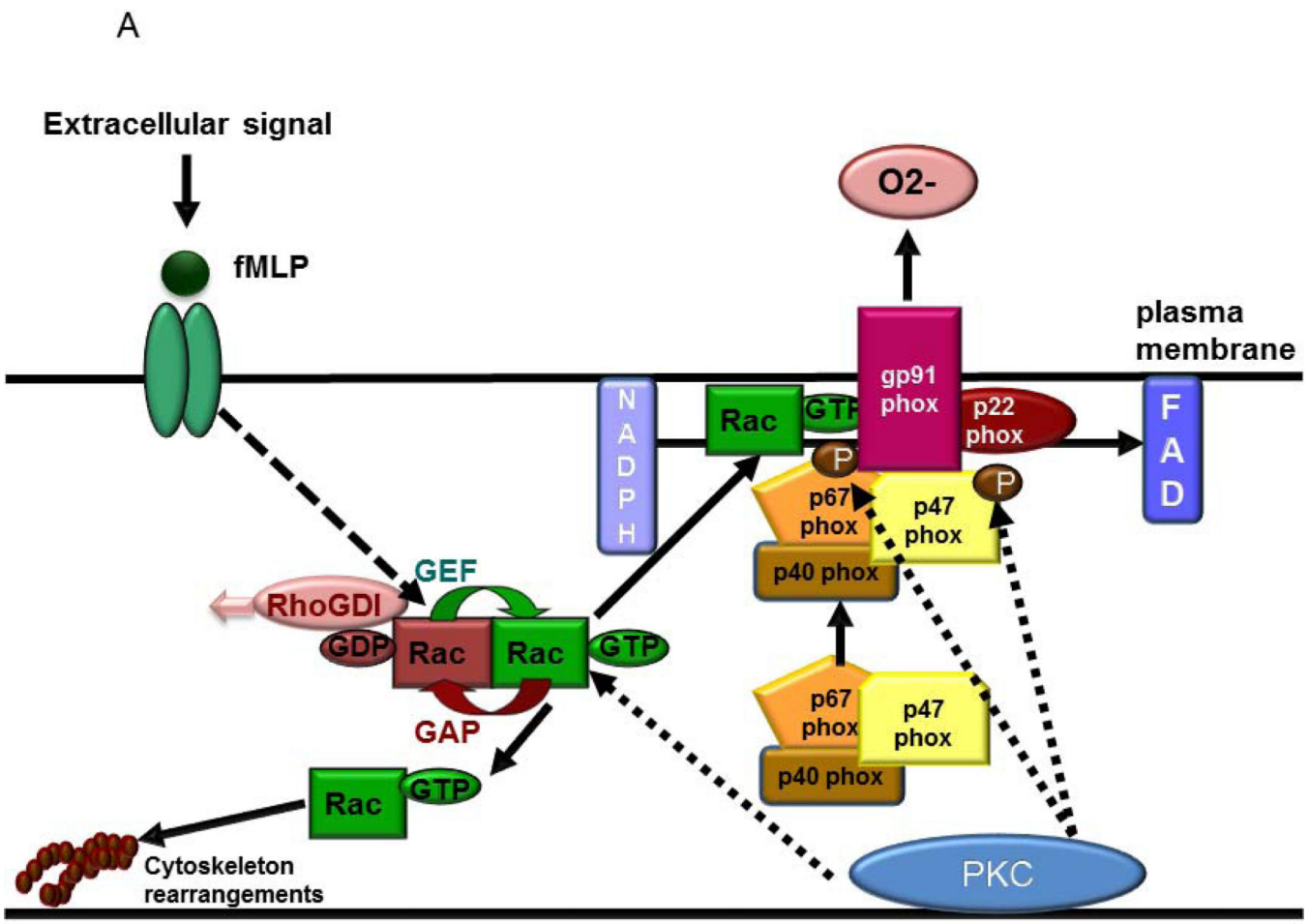

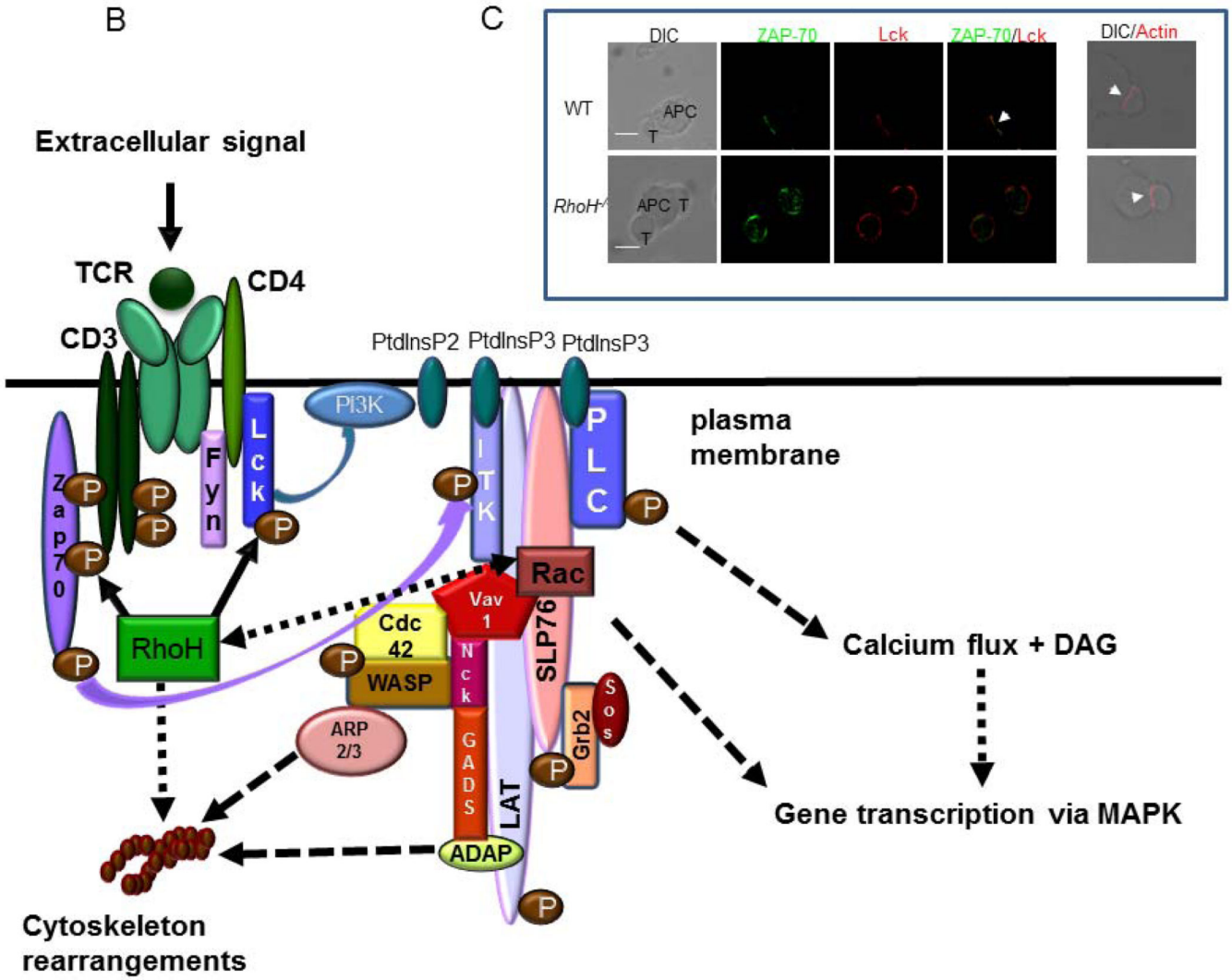

Figure 1. Involvement of Rac in phagocytic cell function and RhoH in T cell receptor signaling

Figure 1A. Involvement of Rac in NADPH oxidase activation and superoxide production and cell migration. Inactive GDP and RhoGDP inhibitor(RhoGDI)-bound Rac is located in the cytoplasm. Activation signals mediated by formylmethionyl-leucyl-phenylalanine (fMLP) binding to its G-coupled receptor, results in dissociation of RhoGDI from Rac and subsequent cycling of Rac to the GTP-bound, active form. Together with $p40^{phox}$, $p47^{phox}$ and the $p67^{phox}$ subunit, activated Rac translocates to the cell or phagosomal membrane to form the active NADPH oxidase complex together with the membrane-bound proteins large glycosylated protein ($gp91^{phox}$) and the smaller adaptor protein $p22^{phox}$. Subsequent electron transfer from NADPH to molecular oxygen via flavin adenine dinucleotide (FAD) leads to formation of superoxide (O2-) radicals. It is postulated that phosphorylation of $p47^{phox}$ and $p67^{phox}$ is mediated by protein kinase C (PKC) and that PKC is also involved in phosphorylation and dissociation of RhoGDI from Rac and thus regulates Rac activation. Activated, GTP-bound Rac also regulates cytoskeleton reorganization via downstream mediators of interaction with F-actin. Lack of Rac therefore disrupts formation of the phagocytic oxidase complex and inhibits migration in phagocytes. (*Figure adapted from*[77]).

Figure 1B. Involvement of RhoH in T cell receptor signaling. The T cell receptor (TCR) complex consists of TCR chains, CD3 and ξ-chain accessory molecules and the co-receptor CD4 or CD8. RhoH is required for phosphorylation of Zeta-chain-associated protein kinase 70 (Zap70) and CD3ξ and recruitment of Zap70 and lymphocyte-specific protein tyrosine kinase (Lck) to the immunological synapse. Thus, in the absence of RhoH the subsequent downstream events including phosphorylation of

IL2-inducible T-cell kinase (ITK) and complex formation mediated by the linker for activation of T cells (LAT) and SH2-domain-containing leukocyte protein of 76 kDa (SLP76) are disrupted. Phosphorylated ITK normally also activates phospholipase C (PLC) which then induces generation of phosphor-inositol-triphosphate (PtdIns) regulating calcium (Ca2+) flux and diacylglycerol (DAG) that activates members of the protein kinase C and RAS guanyl-releasing protein family. These proteins regulate activation of mitogenactivated protein kinases (MAPK) such as JUN amino-terminal kinase, extracellular-signal-regulated kinase 1 and other effectors involved in regulation of gene transcription. Following TCR activation cytoskeleton modifications are also mediated by two downstream pathways that are organized by the LAT-SLP76 complex and thus defective in the absence of RhoH. One pathway depends on degranulation promoting adaptor protein (ADAP), which controls T cell adhesion to the antigen-presenting cell (APC) by up-regulating leukocyte function-associated antigen 1 (LFA1) integrin avidity and the other pathway involves Cdc42, the tyrosine phosphorylated guanine nucleotide exchange factor Vav1, which activates Rac GTPases and controls actin polymerization and TCR clustering through Wiscott-Aldrich syndrome protein (WASP) and the actin nucleating actin related protein (ARP)-complex. Thus, lack of RhoH results in defective TCR signaling, migration and adhesion. Proto-oncogene tyrosine-protein kinase (Fyn), non-catalytic region of tyrosine kinase adaptor protein 1 (Nck), Grb2-like adaptor protein (GADS), growth factor receptor-bound protein 2 (Grb2), Ras guanine nucleotide exchange protein Son of sevenless (Sos), Phosphoinositid-3-Kinase (PI3K) . (*Figure adapted from*[78]).

Figure 1C. Impaired recruitment of Lck to the immunological synapse in *Rhoh*−/− T cells. CD8+ T cells from wt or *Rhoh*−/− p14 TCR transgenic mice were conjugated with gp33 peptide-preloaded APC cells (CH.B2 cells) for 5 min. Cells were fixed and stained with anti-Zap70 (green) and anti-Lck (red). The localization of the immune synapse is indicated with a white arrow. T cell-APC conjugates were fixed and stained with TRITC-Phalloidin for detection of F-actin. Differential interference contrast (DIC) images show the antigen-specific T cell-APC conjugates. In the absence of RhoH localization of Zap70 and LCK to the immunological synapse is impaired, which indicates that RhoH is required for activation and recruitment of Zap70 and Lck to the immunological synapse. (*Figure from*[32]).

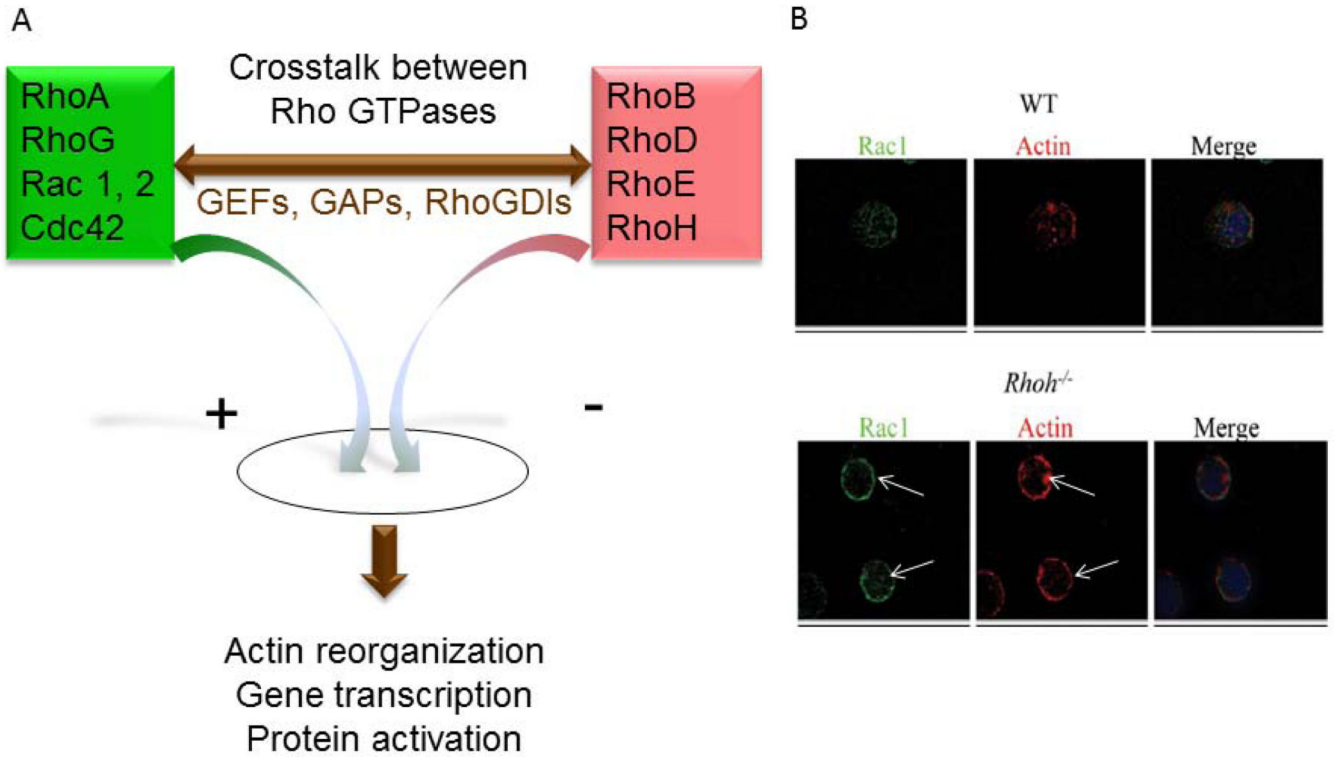

Figure 2. Crosstalk between Rho GTPases

Figure 2A. Crosstalk between GTPases. Members of the RhoGTPase family such as RhoA, RhoG, Rac and Cdc42 appear to have antagonistic effects on cellular functions concerning actin reorganization, gene transcription and protein activation compared to RhoB, RhoD, RhoE and RhoH in various cellular systems. Furthermore it has been demonstrated that members of the Rho GTPase family influence each other directly or via interaction with regulating proteins such as guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) and thus act within an intricate regulatory network. (*Figure adapted from*[79]). An example for this crosstalk is exhibited in Figure 2B in which absence of RhoH is associated with abnormal accumulation of Rac1 in the cell membrane. (*Figure from*[80]).

Figure 2B. RhoH influences subcellular localization of endogenous Rac in hematopoietic stem cells (HSC): Lin-/c-kit- cells (HSC) were fixed and stained with anti-Rac1 mAB (green), rhodamine-labeled phalloidin (red) and 4,6-diamidino-2-phenylindole (blue). Membrane localization is indicated with white arrows. In the absence of RhoH membrane localization of Rac1 is enhanced, which is associated with increased Rac activity, suggesting a regulatory role of RhoH in Rac activation and subcellular localization. (*Figure from*[80]).