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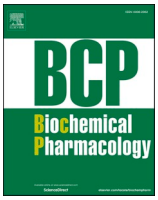

Review

A current overview of RhoA, RhoB, and RhoC functions in vascular biology and pathology

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ABSTRACT

The Rho subfamily members of Rho GTPases, RhoA, RhoB, and RhoC, are key regulators of signal transduction in a variety of cellular processes, including regulation of actomyosin and microtubule dynamics, cell shape, cell adhesion, cell division, cell migration, vesicle/membrane trafficking, and cell proliferation. Traditionally, the focus of research on RhoA/B/C has been on tumor biology, as dysregulation of expression or function of these proteins plays an important role in the pathogenesis of various cancer entities. However, RhoA, RhoB, and RhoC are also important in the context of vascular biology and pathology because they influence endothelial barrier function, vascular smooth muscle contractility and proliferation, vascular function and remodelling as well as angiogenesis. In this context, RhoA/B/C exploit numerous effector molecules to transmit their signals, and their activity is regulated by a variety of guanine nucleotide exchange factors (RhoGEFs) and GTPase-activating proteins (RhoGAPs) that enable precise spatiotemporal activation often in concert with other Rho GTPases. Although their protein structure is very similar, different mechanisms of regulation of gene expression, different localization, and to some extent different interaction with RhoGAPs and RhoGEFs have been observed for RhoA/B/C. In this review, we aim to provide a current overview of the Rho subfamily as regulators of vascular biology and pathology, analyzing database information and existing literature on expression, protein structure, and interaction with effectors and regulatory proteins. In this setting, we will also discuss recent findings on Rho effectors, RhoGEFs, RhoGAPs, as well as guanine nucleotide dissociation inhibitors (RhoGDIs).

1. Introduction

As the inner layer of the vasculature, endothelial cells form an important barrier between the circulating blood and the adjacent tissue. Thus, endothelial cells are important regulators of various physiological processes, such as vascular permeability, leukocyte transmigration, blood cell trafficking, angiogenesis, and vasomotor tone [1]. A multitude of small GTPases are critically involved in the regulation of vascular endothelial cell function. In particular, the Rho GTPase family with its members Rho, Rac and Cdc42 is among the best studied targets in the context of vascular disease pathogenesis and vascular homeostasis [2]. Rho GTPases are GTP-binding proteins (G proteins) that belong to the Ras superfamily of small GTPases. These small sized (~20–30 kDa) monomeric proteins represent the largest class of G proteins, consisting

Abbreviations: ADP, Adenosine diphosphate; Akt, protein kinase B; Arf, ADP ribosylation factors; ARH, aplysia Ras-related homolog; Cdc42, Cell division control protein 42 homolog; DAAM, Dishevelled associated activators of morphogenesis; DLC, deleted in liver cancer; ECT2, Epithelial cell transforming 2; eNOS, Endothelial nitric oxide synsthase; FAK, focal adhesion kinase; FRET, Förster resonance energy transfer; FMNL, formin-like; GAP, GTPase-activating proteins; GDI, Guanine nucleotide dissociation inhibitors; GDP, Guanosine diphosphate; GEF, Guanine nucleotide exchange factors; GPCRs, G protein-coupled receptors; GTP, Guanosine triphosphate; LARG, leukemia associated RhoGEF; mDia, mouse/mammal diaphanous-related formin; miRNA/miR, micro RNA; MLC, regulatory myosin light chains; mRNA, messenger RNA; NO, Nitric oxide; P53, protein with molecular mass of 53 KDa; PDGF, platelet derived growth factor; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PKN, protein kinase N, novel; Rab, Ras-like proteins from rat brain; Rac, Ras-related C3 botulinum toxin substrate; Ran, Ras-related nuclear protein; Ras, Rat sarcoma; Rho, Ras homologous; ROCK, Rho-associated coiled-coil containing kinase; ROS, Reactive oxygen species; siRNA, Small interfering RNA; Src, cellular sarcoma; TNFα, Tumor necrosis factor α; UV, ultra violet; VEGF(-A), Vascular endothelial growth factor; VEGFR-2, Vascular endothelial growth factor receptor 2.

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of more than 150 individual members [3,4]. According to sequence homology, the Ras superfamily can be divided into five different subfamilies: Ras (rat sarcoma), Rab (Ras-like proteins from rat brain), Ran (Ras-related nuclear protein), Arf (ADP ribosylation factors) and the aforementioned Rho (Ras homologous) GTPases [3,4]. In humans, the Rho GTPase family comprises approximately 20 known members that can be subdivided in 8 subfamilies, of which the Rho-subfamily includes the structurally highly similar members RhoA, RhoB, and RhoC [5]. Rho GTPases contribute to cellular processes like cell polarity, cell migration, cell contraction, focal adhesion dynamics, vesicle transport, and cell cycle progression [6]. Within signal transduction pathways, Rho GTPases often link exogenous signals, such as those received via membrane-bound receptors, to effector proteins that regulate either proteins that control the actin cytoskeleton and microtubule dynamics, cell polarity, and vesicle/membrane trafficking or the expression of other proteins [7]. Thereby, Rho GTPases act as molecular switches that enable spatially and temporally defined activation of downstream effector molecules. As a typical feature of G proteins, Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state [8]. The transition to an active GTP-bound state is initiated by proteins upstream of the Rho GTPase that mediate the exchange of GDP to GTP and are therefore referred to as guanine nucleotide exchange factors (GEFs). The resulting conformational changes then allow for interaction with downstream effector proteins. The intrinsic GTPase activity of the protein, which induces the hydrolysis of GTP to GDP, then re-establishes the inactive form of the GTPase and terminates downstream signal transduction. However, compared to other G proteins, the intrinsic GTPase activity of Rho GTPases and other small GTPases of the Ras superfamily is rather weak [9,10]. For timely and proper inactivation, these G proteins must interact with other regulatory proteins that accelerate intrinsic GTP hydrolysis of Rho GTPase and are therefore termed GTPase-activating proteins (GAPs) [11]. In addition, atypical Rho GTPases have been described that are either incapable of GTP hydrolysis or can rapidly switch between GTP- and GDP-bound states [12,13]. For the Rab and Rho family members, another subset of regulatory proteins is known to stabilize the inactive GDP-bound form of Rho GTPases by inhibiting the dissociation of GDP bound to the protein, and are therefore referred to as guanine nucleotide dissociation inhibitors (GDIs) [14]. Like other small GTPases of the Ras superfamily, activated Rho GTPases associate with the plasma membrane to mediate their biological functions. However, it also has been shown that the association with the endomembranes of the cell is important for their function [15,16]. The membrane association is enabled by posttranslational modifications of Rho GTPases at the C-terminus (prenylation with farnesyl or geranylgeranyl, but also palmitoylation) which act as membrane anchors. In addition, proteins that regulate prenylation like the SmgGDS (small GTP-binding protein GDP dissociation stimulator) are also relevant for proper Rho GTPase trafficking [17]. A further function of the aforementioned GDIs is to mask prenylation and protect it from degradation, while also forming a stable complex with the GTPase, thus promoting cytosolic localization of the protein in its inactive state [14,18].

Due to their involvement in metastasis and tumor progression, Rho GTPase are intensively studied in the context of cancer biology [13]. Indeed, Rho GTPases are frequently upregulated in cancer cells and are targets of new anti-cancer therapeutics [19]. However, the Rho GTPases of the Rho subfamily, RhoA, RhoB, and RhoC, are also important for the regulation of endothelial barrier function, as described by multiple groups [20–23]. In this context, it has been shown not only that they differentially affect barrier function [24], but also that changing the expression level of one Rho GTPase causes compensatory changes in the expression levels of the other Rho subfamily members in endothelial cells [25]. Indeed, approaches that directly compare the function of these Rho GTPases in endothelial cells are rare, and it remains to be noted that most aspects of Rho-mediated vascular signalling have been analyzed with a strong focus on RhoA, historically-one of the best studied member of the Rho GTPase family. For instance, RhoA has been shown to be activated by reactive oxygen species (ROS) [26–28]. Vascular oxidative stress itself increases endothelial permeability and adhesion and extravasation of leukocytes [29], implicating that RhoA may act as a central mediator in the development and progression of various cardiovascular pathologies associated with the increased formation of ROS, such as atherosclerosis or aortic aneurysms [30,31]. Another important Rho-dependent regulator of vascular permeability is NO (nitric oxide). Reactive nitrogen species production is controlled by RhoA via regulation of eNOS (endothelial nitric oxide synthase) mRNA stability or expression [32,33], suggesting an important interaction between RhoA and NO signalling with relevance to endothelial homeostasis *in vivo*. Also, inflammatory processes in the vasculature were significantly affected by RhoA and its downstream target ROCK (Rho-associated coiled-coil containing kinase) [34,35]. For instance, adherence and *trans*-endothelial migration of monocytes and neutrophils, depends on leukocyte RhoA and its effector ROCK [34,35]. Additionally, Zhang and colleagues have shown that RhoA and ROCK are involved in numerous atherogenic processes, including cytokine and chemokine release, leukocyte recruitment, oxidised Low-Density Lipoprotein (LDL) uptake into macrophages, and foam cell formation [36]. RhoB has been shown to negatively regulate RhoA activity which is required for endothelial cell migration, angiogenic sprouting and capillary morphogenesis *in vitro* as demonstrated by siRNA-mediated gene knockdown [25]. Interestingly, loss of RhoB was found to decrease pathological angiogenesis in the ischemic retina, whereas RhoB increased lymphangiogenesis after wounding or in the context of inflammation [37]. These results indicate potentially different effects of RhoB in blood vessel versus lymphatic endothelial cells [37]. In the context of vascular inflammation, RhoB activation by TNFα (tumor necrosis factor α), IL1β (interleukine 1β) and LPS (lipopolysaccharide) in endothelial cells has been demonstrated by different groups, a process, in which RhoB seems to be involved in intracellular TNF receptor trafficking [38,39]. Additionally, endosomal RhoB is upregulated in endothelial cells during inflammation in response to inflammatory cytokines [40]. In contrast, the activity of RhoC is not altered by stimulation of endothelial cells with TNFα [38], but it has been described that RhoC activity increases after VEGF (vascular endothelial growth factor) stimulation of endothelial cells *in vitro* [41], a finding that, however, could not be confirmed by other groups using a FRET-based biosensor [36]. Furthermore, knockdown of RhoC using siRNA approaches reduced endothelial cell migration, pseudopodia formation, and angiogenesis, whereas endothelial cell proliferation was enhanced by the absence of RhoC [41,42]. These pro-angiogenic effects of RhoC could be mediated by the activation of a MAPK (mitogen-activated protein kinase)/phospholipase Cγ/Ca^{2+}/eNOS cascade, and the known Rho effector ROCK [41,42].

In this review, we will focus on providing an overview of the function of RhoA, RhoB, and RhoC in the vascular system with a particular focus on the role of the three GTPases in the physiology and pathophysiology of vascular endothelial cells. We will discuss similarities and differences in gene expression, protein sequence, protein structure of these Rho GTPases, while summarizing the current information on these proteins. Here, we will discuss their involvement in endothelial cell homeostasis, angiogenic cell functions, and their role in vascular pathologies linked to (cardio)vascular diseases. To this end, we will also provide an overview of Rho effectors, RhoGEFs, RhoGAPs, and RhoGDIs relevant to vascular and endothelial cell biology (Fig. 1).

2. Transcript structure and regulation of RhoA, RhoB, and RhoC gene expression

In the human genome, the genetic information encoding RhoA, RhoB and RhoC (former gene names: ARH12, ARH6, ARH9, aplysia Ras-related homolog) is located on different chromosomes and differ greatly in their organization of the intron–exon structures.

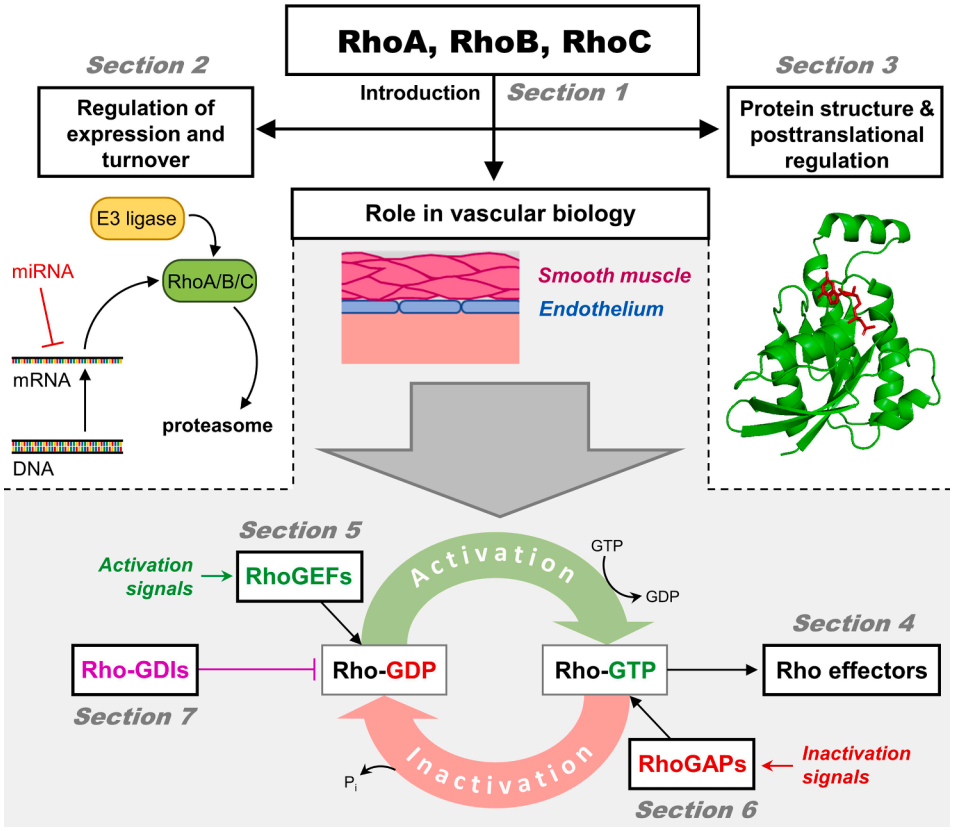

Fig. 1. Overview of RhoA, RhoB, and RhoC functions in vascular biology and pathology. Illustration of the content of this review. Section 1 introduces into RhoA, RhoB and RhoC. Information related to the expression and regulation of expression of RhoA, RhoB and RhoC is covered in Section 2. Protein structure and function of important residues in regulation of RhoA/B/C activity and localization is discussed in Section 3. Function of RhoA/B/C in vascular biology is discussed in Sections 4–7 relating to relevant Rho effector proteins (Section 4), Rho-activating RhoGEFs (Section 5), and Rho-inactivating RhoGAPs (Section 6) and RhoGDIs (Section 7).

Consequently, the mRNA transcripts of RhoA, RhoB and RhoC largely differ in both the total number of transcript variants and their exon composition (Fig. 2). In this respect, the precursor mRNA of RhoA, whose genetic information is located on chromosome 3, is subject to highly variable alternative splicing. Originally, the intron–exon structure of RhoA was described to contain 4 exons only [43]. However, current database information indicates the presence of up to seven different exons spliced into up to seven known transcript variants (Fig. 2A). These transcript variants can be translated into six different RhoA isoforms, of which isoforms 2–6 possess highly truncated RhoA protein sequences. However, the biological relevance of these truncated isoforms is still unclear. In contrast, the genetic information of RhoB is located on chromosome 2, no alternative splicing of RhoB precursor mRNA has been described (Fig. 2B) and consequently only one protein sequence of RhoB is translated. In contrast, the RhoC gene is located on chromosome 1 and alternative splicing of its precursor mRNA gives rise to only three different transcript variants (Fig. 2C). Moreover, all transcript variants of RhoC yield the same protein sequence. Within the open reading frame (ORF), the mRNA transcript sequence of RhoA and RhoB is 71 % identical, whereas the sequence homology of RhoA and RhoC is 78 %. Apart from the mRNA sequence encoding specifically different amino acid residues within the RhoA, RhoB, and RhoC protein structure, the mRNA sequence of the three Rho GTPases within the ORF often differs only in the so-called wobble position of triplets. In contrast, sequence differences in the untranslated regions upstream or downstream of the ORF are much more pronounced and could for example contribute to differential regulation by microRNAs (miRNA).

The expression of RhoA, RhoB, and RhoC in cells is a tightly regulated process that includes transcriptional control by transcription factors, posttranscriptional regulation by miRNAs, and posttranslational regulation by ubiquitin E3 ligases [13,44–46]. At the transcriptional level, RhoA expression is controlled by the cell growth and cell cycle-controlling transcription factor Myc (Myelocytomatosis) in concert with E3 ubiquitin ligase Skp2 (S-phase kinase-associated protein 2) [47], whereas the expression of RhoC is controlled for instance via the cell stress-inducible transcription factor p53 [48]. In contrast, RhoB can be induced by signal transduction pathways controlled by transforming growth factor β (TGFβ) [49] and, independently of p53, in response to genotoxic (e.g. UV radiation) and non-genotoxic stressors (heat, hypoxia, proinflammatory signals like lipopolysaccharides) [50–53]. The expression of RhoA, RhoB, and RhoC is negatively regulated by several miRNAs at the posttranscriptional level, which in turn are controlled by long non-coding RNAs (lncRNAs) [54]. For instance, RhoA expression levels have been shown to be downregulated by miR-133a in cells of the cardiovascular system [55,56] and by miR-125a-3P and miR-155 in other cell types [57,58]. In endothelial cells, RhoB is downregulated by miR-21, resulting in an anti-angiogenic phenotype in miR-21 overexpressing cells [59], whereas overexpression of miR-21 in cancer cells increases their tumorigenic potential by downregulating RhoB-related tumor suppressor activity [60,61]. RhoC is downregulated by miR-106b, miR-138, miR-372, miR-493, and miR-519d in various cancer-related cell types [62–67], thereby reducing their tumorigenic potential, which is why miRNA-based approaches are an interesting option for future individualized cancer therapies. At the posttranslational level, the expression of Rho GTPases is regulated by E3 ubiquitin ligases, which ubiquitinylate the protein and direct it to proteasomal degradation. RhoA and RhoB have been shown to be regulated by E3 ligase Smurf1 (smad ubiquitination regulatory factor 1) [68,69] whose regulatory influence is also important for proper heart development [70]. In addition, RhoA and RhoB can be regulated by the E3 ligase Cullin3, which also plays an important role in vascular homeostasis [46], including endothelial barrier regulation [71]. RhoC has been shown to be regulated by the E3 ligase RNF180 (ring finger protein 180) in gastric cancer cells [72]. In general, dysregulated (over)expression of RhoA and RhoC has been associated with increased tumorigenic potential of malignant cells [73], whereas upregulation of RhoB has been reported to exhibit tumor suppressive potential, depending on the context, due to its function in the cellular response to DNA damage and the associated induction of

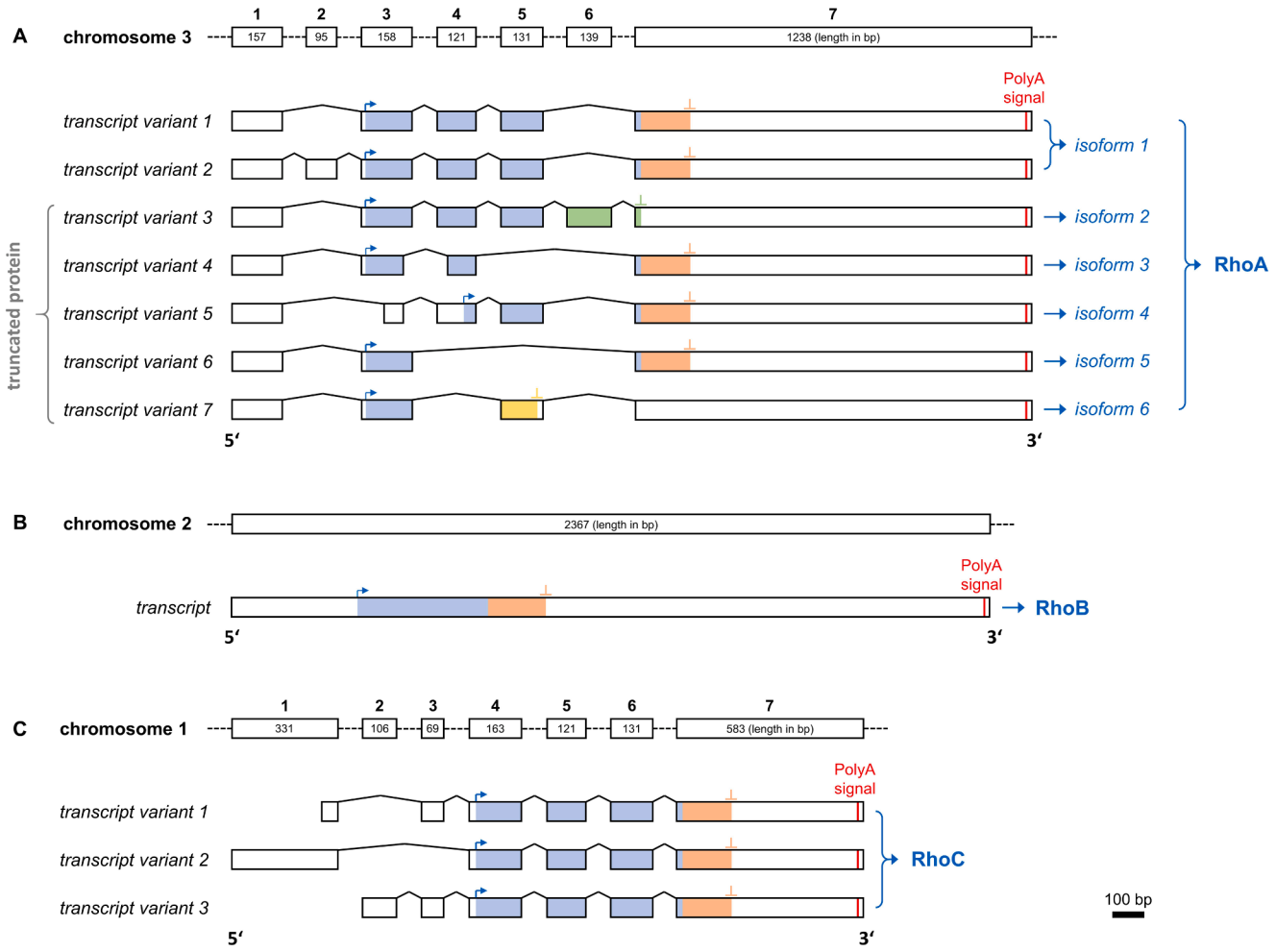

Fig. 2. Genomic structure and known transcripts of human RhoA, B, and C. The human genomic intron–exon-structures of RhoA located on chromosome 3 (A), of RhoB located on chromosome 2 (B) and of RhoC located on chromosome 1 (C) are displayed. The genomic sequences of RhoA and RhoC each comprise seven exons that are alternatively spliced, resulting in seven known transcript variants for RhoA (NCBI accession numbers for transcript variants 1–7: NM_001664.4; NM_001313941.2; NM_001313943.2; NM_001313944.2; NM_001313945.2; NM_001313946.2; NM_001313947.2) and three known transcript variants for RhoC (NCBI accession numbers for transcript variants 1–3: NM_175744.5; NM_001042678.2; NM_001042679.2). The length of the exons is indicated by the number of base pairs within the white boxes representing the exons in the genomic sequence. However, for exon 3 and 4 of RhoA (transcript variants 4 and 5) and exon 1 in RhoC (transcript variant 1), shorter versions of these exons are spliced into the final transcript. RhoB is not subject to alternative splicing, the genetic sequence for the full-length transcript (NCBI accession number: NM_004040.4) can be found on chromosome 2. Within the transcripts, the open reading frames are similarly visualized as in Fig. 3 and their translation results in 6 different protein isoforms for RhoA. Compared to isoform 1, isoforms 2–6 are heavily truncated and most likely do not represent fully active proteins. Only one RhoB protein variant arises from a single transcript and only one RhoC protein variant is translated from three different RhoC transcript variants. The polyadenylation signal of all transcripts is indicated in red at the end of the 3′-untranslated region. Scalebar represents the length of 100 base pairs (bp).

apoptosis [69,74]. Interestingly, the loss of one of the RhoA/B/C GTPases often upregulates expression of the other, suggesting that the upregulation serves functional compensation [75,76].

3. Differences in protein structure of RhoA, RhoB, and RhoC

Most of the information on the protein structure of the Rho subfamily members, has been collected for RhoA, which has been intensively studied for decades. As a result, a large number of RhoA structures have been published over the years showing binding with different nucleotides or nucleotide analogs, fixed in different activity states or interacting with different effectors, GEFs, GAPs, and GDIs (Table 1 with references [77–111]). In contrast, only a few specific RhoB and RhoC structures are available. This could be due to the high importance that RhoA research has had in recent years, but also to the legitimate expectation that all three GTPases are also structurally highly homologous [112]. Indeed, the primary protein sequences of RhoA, RhoB, and RhoC have an even higher sequence homology compared with their mRNA transcripts (83 % for RhoB and 92 % for RhoC compared with RhoA). But how can the differences in signaling between RhoA, RhoB, and RhoC then be explained [24,113,114]?

The Rho GTPases RhoA, RhoB and RhoC possess a relatively conserved *N*-terminal portion containing the G-domain (other names: RhoA-like domain) and a rather less conserved C-terminal tail (Fig. 3) [10]. Within the G-domain, five conserved motifs (G1-G5) are located which are involved in guanosine nucleotide binding and GTP hydrolysis and are highly conserved across the Ras superfamily [115]. Thereby, the G1 motif, also known as P-loop, coordinates the β-phosphate of the guanosine nucleotide and the associated magnesium ion, whereas the G4 and G5 motifs coordinate the interaction with the guanine base. G1-G5 motifs do not differ in RhoA, RhoB, and RhoC (Fig. 3). On the structural level, the G-domain is organized in a six-stranded β-sheet consisting of the five sequence motifs (G1-G5) flanked by six α-helices. Depending on the type of guanosine nucleotide that is bound (GDP or

Table 1
Published protein structures of RhoA, RhoB, and RhoC in the RCSB (Research Collaboratory for Structural Bioinformatics) protein database. For RhoA, only those entries that were referenced in literature were selected.

Accession number	Reso-lution	In complex with	Bound Nucleotide	Notes	Ref.
a) RhoA:					
4XH9	2 Å	Net1 (RhoGEF)	none		[77]
1CC0	5 Å	Rho GDIα	GDP		[78]
3LXR	1.68 Å	Shigella IpgB2	GDP		[79]
3LW8	1.85 Å	Shigella IpgB2	GDP	complex A	[79]
3LWN	2.28 Å	Shigella IpgB2	GDP	complex B	[79]
3T06	2.84 Å	PDZRhoGEF	none	DH/PH fragment	[80]
3KZ1	2.7 Å	PDZRhoGEF	GTPγS[1]	DH/PH fragment	[80]
1XCG	2.5 Å	PDZRhoGEF	none	DH/PH fragment	[81]
6V6V	1.4 Å	–	GDP	RhoA G14V	[82]
6V6U	1.16 Å	–	GDP		[82]
6V6M	1.39 Å	–	GMPPNP[2]		[82]
5JCP	2.1 Å	ARAP3 (RhoGAP)	GDP		[83]
1KMQ	1.55 Å	–	GMPPNP	RhoA Q63L	[84]
1FTN	2.1 Å	–	GDP		[85]
1DPF	2.0 Å	–	GDP	without Mg^{2+}	[86]
6BC0	2.2 Å	p190RhoGEF	GTPγS		[87]
5HPY	2.4 Å	Myosin 9b (RhoGAP)	GDP		[88]
2RGN	3.5 Å	p63RhoGEF, Gαq	GDP	trimeric complex	[89]
4F38	2.8 Å	RhoGDI	GMPPNP	geranylgeranylated	[90]
1A2B	2.4 Å	–	GTPγS	RhoA G14V	[91]
4D0N	2.1 Å	AKAP13 (RhoGEF)	GDP		[92]
5FR1	2.75 Å	RhoGDIα	GDP		[93]
4XSG	1.8 Å	C3cer exoenzyme	GTPγS		[94]
5BWM	2.5 Å	C3cer exoenzyme	GDP		[94]
4XSH	2.5 Å	C3cer exoenzyme	GTP		[94]
7U2P	2.6 Å	TcdA toxin	GDP		[95]
1CXZ	2.2 Å	PKN (PRK1) (Rho effector)	GTPγS		[96]
1S1C	2.6 Å	ROCK1 (Rho effector)	GMPPNP		[97]
6KX2	1.45 Å	–	GDP		[98]
6KX3	1.98 Å	–	GDP	Inhibitor DC-Rhoin	[98]
5FR2	3.35 Å	RhoGDIα	GDP	farnesylated	[99]
1LB1	2.81 Å	Dbl (RhoGEF)	n/a		[100]
6R3V	1.75 Å	RhoGAP domain (not specified)	GDP		[101]
5ZHX	3.5 Å	SmgGDS (RhoGEF)	none	farnesylated	[102]
4XOI	2.09 Å	hsAnillin	GTP	RhoA Q63L	[103]
6BCB	1.4 Å	p114RhoGEF (RhoGEF)	GTPγS		[104]
6BCA	2.0 Å	AKAP13 (RhoGEF)	GTPγS		[104]
5M70	2.2 Å	RhoGAP1 R85A-mutated	GDP		[105]
5M6X	2.4 Å	RhoGAP1 R85A-mutated	GDP		[105]
5IRC	1.72 Å	p190 RhoGAP	GDP		[106]
1OW3	1.8 Å	RhoGAP 1	GDP		[107]
b) RhoB:					
6HXU	1.19 Å	–	GTP	RhoB Q63L	[108]
6SGE	1.5 Å	Nanobody B6	GTP		[108]
2FV8	1.9 Å	–	GDP	without Mg^{2+}	[109]
c) RhoC:					
2GCO	1.4 Å	–	GMPPNP		[110]
2GCN	1.85 Å	–	GDP		[110]
2GCP	2.15 Å	–	GTPγS		[110]
1Z2C	3.0 Å	mDia1 (Rho effector)	GMPPNP		[111]

[1] 5′-Guanosine-diphosphate-monothiophosphate (GTPγS), a stable GTP analog.
[2] 5′-guanylylimidodiphosphate (GMPPNP), a non-hydrolysable analog of GTP.

GTP), the GTPase adopts slightly different protein conformations that establish the active or inactive state, respectively. The conformational changes of both states are mainly confined to two surface loop regions known as switch I and switch II [115]. In these regions, the conserved G3 motif (switch I) and G4 motif (switch II) are located. In the active, GTP-bound state, the switch II region coordinates a water molecule which is needed for the nucleophilic attack for the hydrolysis of the γ-phosphate of GTP. The conformational changes in switches I and II from the inactive GDP-bound to the active GTP-bound state are also a prerequisite for the GTPase to bind effector proteins, which likely occurs through interaction with hydrophobic residues in switch regions that are exposed in the active state of the GTPase [116]. On the level of protein sequence, the only differences within the switch regions are residue 29 which is glutamate in RhoB and glutamine in RhoA/RhoC and residue 43, which is isoleucine in RhoC and valine in RhoA/RhoB. Although being both similarly aliphatic, the difference in residue 43 has been related to the inability of RhoC to be activated by the RhoGEF XPLN (exchange factor found in platelets, leukemic, and neuronal tissues) [117]. Interestingly, mutation of RhoA, RhoB, and RhoC at this residue had differential effects on the nucleotide exchange rates of various GEFs, suggesting that it is a critical residue for GEF activity but not necessarily for GEF selectivity [118]. In addition to the switch regions, there is an insert region, a RhoA/B/C-characteristic α-helix, that has also been shown to be of importance for the interaction of the Rho GTPases with GEFs and downstream effectors [10,119]. Within the insertion region, several differences are found between the protein sequences of RhoA, RhoB, and RhoC that are likely to be structurally relevant to the selectivity of Rho GTPases towards GEFs and downstream effectors. In addition, the hypervariable C-terminal tail largely differs between RhoA, RhoB, and RhoC. The predominantly basic, positively charged residues of the hypervariable tail of RhoA/RhoC compared with the predominantly polar residues of this region of RhoB were held responsible for the selective

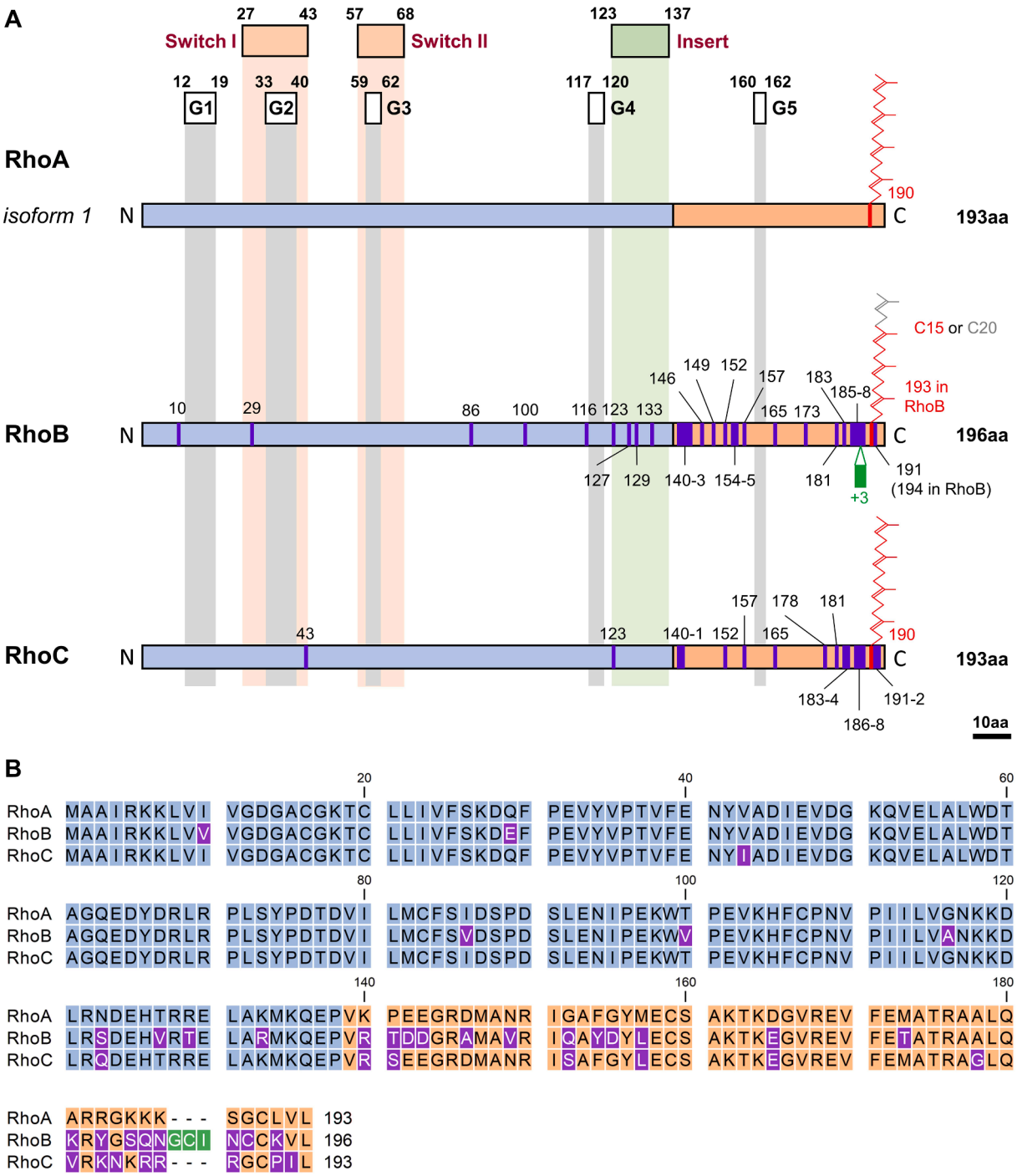

Fig. 3. Protein sequence organization of human RhoA, RhoB, and RhoC. A) The protein sequence of RhoA isoform 1 (NCBI accession number: NP_001300870.1), RhoB (NCBI accession number: NP_004031.1), and RhoC (NCBI accession number: NP_786886.1) were mapped against the RhoA sequence. The protein sequence is structured into a relatively constant and conserved *N*-terminal part (blue) and a less well conserved C-terminal tail (orange). Based on information from Schaefer et al. [10], the position of the conserved sequence motifs G1-G5 as well as switch regions I and II and the insert region are indicated at the top of the figure. Small numbers indicate the respective position of amino acids within the protein sequence in relation to RhoA isoform 1. For RhoB and RhoC, the position of differences in the protein sequence is indicated in purple with the number of the changed amino acid residue in relation to RhoA isoform 1. RhoB has an insert of 3 amino acids (green) in its C-terminal tail, resulting in a slightly longer protein sequence, as indicated by the total number of amino acids in the sequence on the right side of the figure. Prenylation at cysteine residue 190 (geranylgeranylation of RhoA, RhoC) or 193 (geranylgeranylation or farnesylation of RhoB) is indicated in red. Scalebar represents the length of 10 amino acids (aa). **B)** Direct comparison of the total amino acid sequence between RhoA, RhoB and RhoC with *N*-terminal part (blue) and C-terminal tail (orange). Residues in RhoB and RhoC that differ from RhoA sequence are highlighted in purple, the 3 amino acid insert in RhoB is highlighted in green.

interaction of the RhoGEF SmgGDS (small GTP-binding protein GDP dissociation stimulator) with RhoA and RhoC [120]. To date, however, not more is known about the structural basis of the selectivity of RhoA/B/C for specific effectors, GAPs, or GEFs. Moreover, most of the relevant differences of RhoA, RhoB, and RhoC in binding GEFs and effectors might be mainly due to the different subcellular regulation and distribution of Rho GTPases, which restricts their interactions to locally available GEFs, GAPs, and effectors while allowing appropriate spatiotemporal signaling [10,121].

There are critical residues in the protein sequences of RhoA, RhoB, and RhoC that are frequently mutated to modulate Rho function, providing a tool to study Rho-related effects. The G14V and Q63L mutations have been shown to disrupt GTP hydrolysis and lead to persistent GTP binding, resulting in a constitutively active form of the GTPase [122]. In contrast, the T19N mutation mimics the inactive state giving rise to a dominant-negative form of the GTPase that is incapable of activating downstream effectors [123]. Furthermore, the protein sequence of RhoA is target of several regulatory kinases phosphorylating the protein at residues Ser26, Ser88, Thr100, Thr127 and Ser188, most of which have regulatory effects on activation state, expression, or localization [124]. Ser26 and Ser88 are conserved within RhoA, RhoB, and RhoC, and Thr100 and Thr127 are conserved within RhoA and RhoC. However, phosphorylation at these sites has not been demonstrated so far for RhoB and RhoC, suggesting differences between RhoA, RhoB, and RhoC with respect to these particularities of posttranslational regulation. Accordingly, phosphorylation by Chk1 (checkpoint kinase 1) at Thr173 and Thr175, by CK1 (casein kinase 1) at Ser185 for RhoB [125–127], and by Akt at Ser73 for RhoC has been demonstrated [128,129]. In addition to Ser/Thr phosphorylation, also phosphorylation at tyrosine residue Tyr42 has been reported for RhoA, recently [130–132]. This phosphorylation is mainly induced by the small non-receptor tyrosine kinase Src (cellular sarcoma) in a redox-sensitive manner and induces the translocation of phosphorylated RhoA to the nucleus, where it has been shown to act as transcriptional regulator for promoter regions of vimentin and NAD kinase [131,132]. Aside from phosphorylation, RhoA, RhoB and RhoC also undergo posttranslational prenylation that is important for their membrane localization. At the C-terminal cysteine residue Cys190, RhoA and RhoC are geranylgeranylated, whereas RhoB is either farnesylated or geranylgeranylated at the corresponding Cys193, possibly directing the Rho GTPases to different cellular compartments (RhoA, RhoC to the plasma membrane, RhoB to endosomal membranes) [112]. In addition, palmitoylation of RhoB has been described [133].

4. Effectors in RhoA-, RhoB-, and related signaling

Rho effectors mediate downstream signaling of Rho GTPase and are activated by direct binding to their GTP-bound active state. About 30 effectors of Rho GTPases have been identified that play a role in many different biological processes [134]. In this context, most effectors are not limited to activation by a specific Rho GTPase, but can often be activated by different Rho GTPases. Thereby, Rho effectors can function as kinases that phosphorylate and activate/inactivate other downstream targets, as scaffolding proteins that contribute to compartmentalization of signaling processes within a cell, or as proteins (e.g. formins) that directly interact with and regulate the actin cytoskeleton. Especially Rho effectors that act as kinases have the ability to amplify signaling processes by simultaneously regulating a multitude of other downstream targets, that include actomyosin, microtubule, and intermediate filament regulators, transcription factors, scaffolding proteins, and regulatory proteins of cross-talking signaling pathways [135–138].

One of the best characterized Rho effectors is ROCK (Rho-associated coiled-coil containing kinase), of which there are two isoforms, ROCK1 and ROCK2. ROCK specifically interacts with RhoA, RhoB, and RhoC via its Rho-binding domain, thereby tethering to the switch regions (switch 1 / 2) of the activated Rho GTPases [97]. Consequently, the kinase activity of ROCK is activated to phosphorylate multiple downstream targets [139]. No differences in the affinity of ROCK1 and ROCK2 to RhoA, RhoB, or RhoC have been described, and ROCK1 and ROCK2 appear to possess similar kinase activities. Nevertheless, they exhibit differences in terms of their subcellular localization and expression regulation in various cell types [139]. In this context, specific knockdown of ROCK1 and ROCK2 has shown that both kinases can induce different biological effects. It has been shown that selective silencing of ROCK1, similar to silencing of RhoA but different from silencing of RhoC, altered cell shape, stress fibers, and focal adhesion formation, whereas selective silencing of ROCK2 impaired cellular protrusion formation, phagocytosis, and cell contraction, results suggesting distinct roles of ROCK1 and ROCK2 in RhoA and RhoC signaling [114,140,141]. Important downstream targets of ROCK for actin reorganization include MLCP (myosin light chain phosphatase) and regulatory myosin light chains (MLC), which induce cross-linking of F-actin bundles (stress fibers) with myosin (e.g., nonmuscle myosin II) and generate contractile force along these bundles. In addition, the ROCK downstream targets LIMK1 and LIMK2 (lin11 isl-1 mec-3 motif containing kinase) inactivate the actin depolymerizing enzyme cofilin (also ADF, actin depolymerizing enzyme), thereby increasing stress fiber stability [142]. In addition, LIMK1/2 also appear to regulate microtubule networks [142]. Stress fibers are connected to focal adhesions of the adherent cells, which connect the cells to the extracellular matrix. Rho/ROCK-induced cell tension is conveyed via stress fibers to focal adhesion, thereby drastically changing their composition [143] and priming the cell for cell contraction. In addition, ROCK activates its downstream effector and tumor suppressor PTEN (phosphatase and tensin homologue). ROCK-activated PTEN counteracts the function of PI3K (phosphoinositide 3-kinases), thereby inhibiting Akt signaling pathways that regulate cell growth and proliferation, but also eNOS activity and consecutive NO production of the vascular endothelium [144]. In the vasculature, RhoA-driven ROCK signaling has a number of implications for endothelial barrier function, endothelial cell motility, angiogenesis, vasotonus, and for the development of endothelial dysfunction and hypertension [145–150]. In addition, Akt-mediated expression and phosphorylation of eNOS at Ser1179 is reduced thereby, impairing endothelial cell-driven NO-related effects on vascular tone and homeostasis [20].

Citron Rho-interacting kinase (Citron-K) [151] and its shorter relative lacking the kinase domain, Citron-N, which is most abundant in neuronal cells [152], are other important Rho effectors. RhoA signaling via Citron-K is required for cytokinesis, one of the last steps of cell division, in which a contractile ring of actin-myosin is formed to separate the cytoplasm of the dividing cell into two distinct cells (abscission) [153,154]. Similar to ROCK, Citron-K has been shown to be able to phosphorylate regulatory MLC of Myosin II [155], although the precise role of Citron-K during this step is controversial [156]. However, Citron-K interacts with Anillin, a scaffolding protein that is recruited to the equatorial membrane during cytokinesis and is directly involved in contractile ring formation [157]. Anillin also bears a RhoA binding domain and is therefore also an effector of RhoA during cytokinesis. Being part of cell cycle regulation, silencing of Citron-K has been shown to decrease the tumorigenic potential of cancer cells, while high expression of Citron-K has been associated with poor prognosis in cancer treatment [158,159]. In addition to cell division, another role of Citron-K was demonstrated in phosphorylation of Thr497 of eNOS, a phosphorylation that inactivates the enzyme and may be involved in the pathogenesis of vascular dysfunction after arsenate treatment [160].

Another subset of kinases that act as downstream effectors of RhoA/B/C is the protein kinase N (PKN, N for novel) subset also termed protein kinase C-related kinase (PRK). Three isoforms of this kinase exist (PKN1 = PKNα = PRK1, PKN2 = PKNγ = PRK1, PKN3 = PKNβ = PRK3). All isoforms of PKN can bind to RhoA/B/C via their homology repeat 1a (HR1a) domains and HR1ab di-domains with different affinities, thereby showing highest binding affinities of all kinases to RhoB [161]. In addition to Rho, PKN1 is mainly activated by 3-phosphoinositide-

dependent protein kinase 1 (PDK1), with Rho binding prior to PDK1 phosphorylation being able to enhance PKN1 activity [162]. Via this route RhoA/B/C is again connected to the PI3K/PDK1/Akt signaling pathway. In the vascular system, PKN1, an effector of RhoA and Rac1, has been shown to be involved in vascular wall remodeling [163] by influencing aortic smooth muscle cell growth and migration after balloon catheter-induced blood vessel injury [164]. PKN1 and PKN2 induce immediate early genes like the proto-oncogene c-Fos, which is part of the transcription factor AP-1 (activator protein 1), resulting in the expression of proteins relevant for mitosis or migration of smooth muscle cells [165]. In addition, PKN1 is involved in the regulation of neutrophil adhesion to endothelial cells by triggering polarized RhoA activity in neutrophils and directing trafficking of integrins [166]. PKN2 activity has been shown to phosphorylate eNOS at Ser1179 (similar to Akt phosphorylation) to activate NO production of endothelial cells in response to laminar flow, thereby decreasing vascular tone via NO-induced smooth muscle cell relaxation [167]. Although, RhoA was not directly involved in this process, as RhoA knockdown did not alter flow-induced PKN2 activation, RhoA may be involved in long-term effects such as structural changes in the cytoskeleton in response to shear stress [168]. In addition, PKN2 knockout mice are lethal and display major defects in cardiovascular architecture, which may indicate the relevance of the RhoA-PKN2 signaling axis during fetal development [169]. Similar to Citron-K, PKN2 has also been shown to be involved in the regulation of cytokinesis [170]. RhoC-mediated PKN3 signaling in the vascular endothelium has been shown to be a major regulator of tumor angiogenesis and cancer progression [171–174], whereas depletion of PKN3 in endothelial cells impaired their morphology, disrupted cell–cell junctions and reduced motility and the angiogenic potential these cells [175].

Aside from regulatory kinases, there are also Rho effectors that directly regulate the actin / microtubule network. Among them, one type is diaphanous-related formins (DIAPH / DRF, mDia in mouse/mammals), which have been shown to interact with switch regions of the Rho GTPase RhoC via its GTPase binding domain (GDB) and formin homology domain 3 (FH3) [111]. The three isoforms of mDia have been shown to interact with different Rho GTPases including RhoA, RhoB, and RhoC. Thereby, mDia1 (equivalent to DIAPH1 / DRF1 in humans) can be activated by RhoA, RhoB, and RhoC [111], whereas mDia2 (equivalent DIAPH3 / DRF3 in humans) can be activated by RhoA and RhoB but not by RhoC [176]. mDia3 appears to binds to RhoA but may act mainly through other GTPases like Cdc42 and Rac1 [177]. As a formin, mDia has the ability to nucleate monomeric G-actin at membranes and elongate polarized, unbranched F-actin filaments. Therefore, mDia is an important factor in the formation of new actin filaments and contributes to actin rearrangement in close interaction with the aforementioned Rho effector ROCK [178–180]. In this regard, mDia1-3 have been shown to be involved in actin-related processes such as filopodium formation, cell shape, cell polarity, mechanotransduction, cytokinesis, and migration, as well as in processes of microtubule organization [178,180–183]. While mDia-related functions at the plasma membrane are associated with its activation by RhoA and RhoC, RhoB is thought to regulate mDia1/2 at endosomal membranes and, via this, also plays an important role in membrane trafficking [184,185].

Another type of important diaphanous-related formins that act as Rho effectors are the proteins FMNL1, FMNL2 and FMNL3 (formin-like). FMNL1 is known to be activated downstream of Cdc42, however, FMNL2 and FMNL3 act downstream of RhoC but apparently not downstream of RhoA and RhoB [114,186]. FMNL formins have been shown to bind and bundle actin filaments via their FH2 domain and to connect actin filaments to microtubule-organizing centers [187]. In addition, RhoC signaling via FMNL2/3 has been shown to regulate cell migration, cell invasion and lamellipodia broadening [114,186]. Furthermore, RhoC signaling via FMNL3 has been shown to regulate the activity of focal adhesion kinase (FAK) and the assembly of plasma membrane protrusions that contribute to cell–cell adhesion [188,189].

Another Rho effector which belongs to the formin protein family is DAAM (Dvl or Dishevelled associated activators of morphogenesis). Until now, two isoforms, Daam-1 and Daam-2, are known that have been shown to be part of a non-canonical, β-catenin-independent Wnt (wingless / integrated) signaling pathway [190]. In general, the Wnt signaling pathway is critically involved in defining planar cell-polarity and cell axis in almost all cells including cells of the vascular system [191,192]. Indeed, Daam-1 and Daam-2 play a crucial role in cardiac development by regulating cardiac muscle maturation and sarcomere assembly, processes that, however, do not depend on upstream RhoA activation [190]. Similarly, in humans, inactivating mutations of DAAM-1 have been associated with congenital heart defects, suggesting a critical role in cardiac development [193], findings that are further supported by additional evidence from Daam1-deficient mice that exhibit embryonic and neonatal lethality and suffer from multiple cardiac defects [194]. In contrast, Rho-related effects of DAAM may be of greater importance in cells of the adult organism. Similar to other formins, DAAM-1 contains a FH2 domain which directs nucleation and elongation of new actin filaments [195]. In this regard, DAAM-1 was found to be localized along filopodial shafts, where it bundles actin filaments and cooperates with Fascin to drive filopodia formation [196]. In platelets, DAAM-1 has been shown to interact with RhoA, RhoB, and RhoC and to be activated after thrombin-induced RhoA signaling [197]. Furthermore, immunohistochemical analysis of lung tissue sections showed increased expression levels of DAAM-1 in endothelial cells, myofibroblasts, and medial smooth muscle cells of elderly patients suffering from idiopathic pulmonary arterial hypertension compared with age-matched healthy controls [198]. In the context of vascular biology and pathophysiology, much less is known about DAAM-2. However, certain variants of DAAM-2 or its overexpression have been associated with actin dysregulation in nephrotic syndrome and podocytic diabetic nephropathy [199,200].

In addition to the above effectors, there are several known RhoA/B/C effectors whose role in vascular (patho)physiology has not yet been investigated according to the literature. These include the Rho effector and scaffolding protein Rhotekin (RTKN1&2), which is predominantly expressed in brain, kidney, lung, and skeletal muscle (RTKN1) or lymphocytes (RTKN2) and thus has been studied mainly in the context of the pathogenesis of cancer or neuronal pathologies [201]. Similarly, the Rho effector and scaffolding protein Rhophilin (RHPL1&2), which is thought to bind RhoA and RhoB, has not yet been found to be of significant importance in the pathogenesis of vascular disease [202–204]. Rhophilin-1 is most abundant in testis [205] whereas Rhophilin-2 is ubiquitously expressed and both isoforms can interact with Rho GTPases in a nucleotide-independent fashion [202]. Although effects on the organization of the cytoskeleton have been demonstrated, the mechanism of their interaction and interplay with RhoA in the cellular context remains largely unclear.

5. RhoA,B,C related RhoGEFs involved in vascular biology and pathology

As mentioned earlier, RhoGEFs are activators of Rho GTPases by inducing the exchange of GDP to GTP, which enables Rho GTPases to activate downstream effectors. However, RhoGEFs are also regulated themselves in their expression, phosphorylation state, and localization in the cell, providing further fine-tuning of cellular Rho activation [206,207]. In this context, RhoGEFs are often localized near their target structure in the cell and often elicit only a spatially restricted Rho response [208–210]. Thus, in the cell, Rho-specific GEFs are not restricted to the plasma and endosomal membranes, but can also be enriched or present at specialized structures such as actin and myosin filaments, cell junctions, focal adhesion, or the nucleus. The number of known RhoGEFs exceeds the number of regulated Rho GTPases by a factor of three [211]. Some RhoGEFs specifically activate only a single Rho GTPase, whereas other RhoGEFs are less selective and can induce

the activity of several Rho GTPases. In many cellular processes, multiple RhoGEFs closely cooperate to enable spatiotemporal activation of Rho GTPases, which increases complexity and consequently makes elucidating the underlying signal transduction pathways their cellular consequences more difficult [208–210]. Currently, 81 individual members of the RhoGEFs protein family have been identified, which are also expressed at varying levels in endothelial cells [212]. The common gene names of RhoGEFs often have a unified nomenclature with ARHGEF (derived from the original name of Rho GTPases: Aplysia Ras-related Homolog, GEF for guanine nucleotide exchange factor) followed by an ordinal number. RhoGEFs that have been shown to activate RhoA, RhoB, and RhoC are summarized in Table 2 (with references [92,117,118,120,213–243]). However, it should be noted that most RhoGEFs were only investigated and tested in the context of RhoA activation. Given the high structural similarities, many of these RhoGEFs could in principle also be candidates for RhoB and RhoC activation.

In general, RhoGEFs can be subdivided in two classes depending on the nature of their RhoGEF domain. The majority of RhoGEFs contains at least one tandem of a Dbl-homology domain (DH, Dbl … diffuse B-cell lymphoma) and a pleckstrin-homology domain (PH) and are referred to as DH/PH class or the Dbl family of RhoGEFs, comprising approximately 70 members [244,245]. This DH/PH tandem is mediating the interaction with Rho GTPases to induce the exchange of GDP to GTP, thereby converting the protein to its active form [246]. The second class of RhoGEFs contains a DOCK homology region 2 (synonym: Docker2) that serves similar functions as the DH/PH tandem without having a high level of sequence homology [247]. This RhoGEF domain is only found in proteins of the DOCK family (dedicators of cytokines) which contains 11 members and is known to activate Rac and Cdc42 but not RhoA, RhoB, or RhoC [248]. A well-known subfamily of DH/PH class RhoGEFs is the RGS family (regulator of G-protein signaling), which has an *N*-terminal RGS or RH domain (RGS homology) in addition to a DH/PH tandem [249–251]. This domain is able to interact with the α-subunit of heterotrimeric $G_{12/13}$-proteins that are activated by certain G protein-coupled receptors (GPCRs), such as the thromboxane A_2 receptor (TBXA2R) [146]. There are three known members of RGS family RhoGEFs: p115RhoGEF (=ARHGEF1), PDZ-RhoGEF (postsynaptic density 95/disk-like/zonula occludes 1 RhoGEF = ARHGEF11) and LARG (leukemia associated RhoGEF = AHRGEF12) [249–251]. p115RhoGEF has been shown to activate RhoA via G_{13} [251], whereas LARG, depending on its phosphorylation state, is activated by G_{13} or G_{12} and subsequently induces RhoA activity [252]. PDZ-RhoGEF also activates RhoA via G_{13} [253], however, also coupling to G_s has been suggested for PDZ-RhoGEF, which could redirect signal transduction primarily towards the activation of Cdc42 [250]. Besides the RGS family, also other RhoGEFs that do not contain an RGS/RH domain, such as Lbc-RhoGEF (lymphoid blast crisis RhoGEF, AKAP13) and p114RhoGEF, can also be specifically activated by heterotrimeric $G_{12/13}$ proteins via an unknown mechanism [254]. To date, approximately 30 GPCRs are known to transduce signals via $G_{12/13}$ and their associated RhoGEFs [255–257], many of which are also relevant in the context of vascular biology. In this respect, the mainly G_q-coupled AT_1 receptor (angiotensin II receptor subtype 1) [258] has been shown to transduce Rho/ROCK signals via G_{12}, contributing to vasoconstriction of vascular smooth muscle cells and thus influencing systemic blood pressure [259]. Similarly, the thromboxane A_2 receptor and the thrombin receptors PAR1, PAR3 and PAR4 (proteinase activated receptors), known for their involvement in platelet aggregation, also transduce RhoA signals via G_{13} [260,261]. Through this, both thrombin and thromboxane A_2 are able to stimulate contraction of vascular smooth muscle cells [262,263] and restrict blood flow in injured blood vessels during primary hemostasis. Interestingly, in endothelial cells, thromboxane A_2 receptor and G_{13}-mediated activation of both RhoA and RhoC can jointly trigger anti-angiogenic signal transduction [146]. Similar effects on angiogenic capacity were also observed in endothelial cells in which a constitutively active variant of RhoA were expressed [33].

Table 2
RhoGEFs known to be relevant to RhoA, RhoB, and RhoC signaling. Gene names were assigned according to the NCBI database. RhoGEFs with known functions in vascular cells are highlighted in bold.

Gene name	Common protein names	Involved in	RhoA	RhoB	RhoC
ARHGEF1	**P115RhoGEF**	**GPCR ($G_{12/13}$) mediated signaling**	[213]	[213]	[213]
ARHGEF2	**GEF-H1**	**Microtubule depolymerization**	[214]	[215]	–
ARHGEF3	XPLN	Skeletal muscle regeneration, platelet differentiation	[117]	[117]	no
ARHGEF5	Ephexin3, TIM, Scambio	Ephrin receptor mediated signaling Tumorigenesis, neuronal development, Src-induced podosome formation	[216,217]	–	[217]
NET1	ARHGEF8	Cell survival, cell motility, regulation of apoptosis	[218]	[219]	[220]
ARHGEF10	**GEF10**	**Vesicle trafficking, endothelial cell barrier**	[221]	[222]	–
ARHGEF10L	GrinchGEF	Tumorigenesis	[223–226]	–	–
ARHGEF11	**PDZRhoGEF**	**GPCR ($G_{12/13}$)-mediated signaling**	[213]	[213]	[213]
ARHGEF12	**LARG**	**GPCR ($G_{12/13}$)-mediated signaling**	[213]	[213]	[213]
AKAP13	**Lbc-RhoGEF, AKAP-Lbc, ARHGEF13**	**GPCR ($G_{12/13}$)-mediated signaling**	[92]	[227]	[227]
MCF2L	DBS, ARHGEF14	Tumorigenesis, Ephrin receptor-mediated signaling	[118]	[118]	[118]
ARHGEF15	**Ephexin5, Vsm-RhoGEF**	**Ephrin receptor-mediated signaling, Dendritic spine growth**	[228]	–	–
ARHGEF17	**TEM4**	**Cell junction formation, cell migration**	[229]	[229]	[229]
ARHGEF19	Ephexin2, WGEF	Ephrin receptor signaling, intestinal Tumorigenesis	[230,231]	–	–
MCF2	DBL, ARHGEF21	Tumorigenesis	[118,232]	[118]	[118]
TRIO	**ARHGEF23**	**GPCR (G_q)-mediated signaling**	[233]	–	–
ARHGEF25	**p63RhoGEF / GEFT**	**GPCR (G_q)-mediated signaling**	[234]	[234]	[234]
NGEF	Ephexin1, ARHGEF27	Ephrin receptor-mediated signaling, Neuronal cells	[235]	–	–
ARHGEF28	**p190RhoGEF**	**Endothelial cell focal adhesion formation & migration, tumorigenesis**	[213]	[213]	[213]
OBSCN	Obscurin, ARHGEF30	Striated muscle contractility, tumorigenesis	[236]	–	–
ECT2	**Ect2, ARHGEF31**	**Diapedesis, cytokinesis, angiogenesis,** regulation of apoptosis	[237]	[219]	–
ARHGEF40	**SOLO**	**Stretch-induced reorientation, migration, mechano-sensing**	[238]	–	–
PLEKHG5	**Syx, Tech**	**Regulation of endothelial cell tight junction, migration, angiogenesis**	[239]	–	–
PLEKHG6	MyoGEF	Neurogenesis & neural migration, cancer cell invasion	[240]	–	[241]
RAP1GDS1	SmgGDS	Rho prenylation & trafficking	[120]	no	[120]
VAV1	**VAV**	T/B-cell receptor signaling in hematopoietic cell, **endothelial cell migration, angiogenesis, tumorigenesis**	[242]	–	–
VAV2	**VAV-2**	**VEGFR2 signaling, endothelial cell migration, angiogenesis, tumorigenesis**	[118]	[118]	[118]
VAV3	**VAV-3**	Hematopoietic cell, **endothelial cell migration, angiogenesis, tumorigenesis**	[243]	–	–

In addition to GPCRs that induce $G_{12/13}$-mediated activation of Rho GTPases, it has been shown that GPCRs that couple to the $G_{q/11}$ family of heterotrimeric G proteins and thus activate primarily phospholipase C-β signaling can also induce the activity of Rho GTPases via $G_{q/11}$-dependent signaling [264,265]. In this respect, the three different isoforms of ARHGEF25, p63RhoGEF^{580}, GEFT (guanine nucleotide exchange factor T), and p63RhoGEF^{619} have been shown to activate RhoA, RhoB, and RhoC by direct interaction with G_q similar to RGS RhoGEFs that activate $G_{12/13}$ [234,266]. Since ARHGEF25 is also present in vascular smooth muscle cells, signaling from RhoA via G_q provides an alternative pathway to induce contraction of these cells and increase vascular tone [267]. In this respect, increased levels of ARHGEF25 have also been associated with hypertension [268]. Another RhoGEF which has been shown to transduce RhoA signaling via $G_{q/11}$ is TRIO, which is named after its three enzymatic domains [234,269]. This RhoGEF bears two different GEF domains that can activate either Rac1/RhoG (GEF1 or TrioN) or RhoA (GEF2 or TrioC) [270] and that can be composed differently in the 6 known isoforms of TRIO (TRIO and TRIOA-E) [271]. TRIO was shown to be involved in transendothelial migration of leukocytes (diapedesis) [271,272]. In this context, the expression TRIO together with various cell adhesion molecules is upregulated in endothelial cells upon inflammatory stimuli (e.g. TNFα) [273]. One of these adhesion molecules is ICAM-1 (intracellular adhesion molecule 1), which facilitates the binding of leukocytes to endothelial cells by clustering at their contact site and forming a so-called docking structure by local actin polymerization [271,272]. TRIO, which is recruited to the ICAM-1 clusters, is instrumental in the assembly of the docking structure and mainly regulates Rac1 and subsequent RhoG activation via its GEF2 domain [272]. In addition also other cell contacts, for example between endothelial and vascular smooth muscle cells and between endothelial cells and pericytes via *N*-cadherin (neuronal cadherin) can induce signaling from RhoA via TRIO, resulting in an increased cell tension [274]. In endothelial cells, the $G_{q/11}$ pathway is also activated in response to shear stress independently of receptor activation, which presumably provides a way for cells to signal via mechanotransduction [275]. This activation of $G_{q/11}$ signaling could potentially activate RhoA signaling through TRIO, as previous studies indicated that the GEF activity of TRIO is autoinhibited and that autoinhibition is abolished upon $G_{q/11}$ binding [269]. However, another known mechanosensor in endothelial cells, VE-cadherin (vascular endothelial cadherin), has also been reported to accumulate at cell–cell junctions along with TRIO when laminar flow is initiated [276]. Consequently, TRIO has been shown to be required for flow-induced endothelial cell alignment by serving as a Rac1 scaffolding protein to induce polarization of cells in the flow direction without directly activating it [276]. However, both, activated RhoA and Rac1 were observed during the application of shear stress [277], possibly indicating the involvement of other RhoGEFs in mechanosensitivity. On the level of GPCR-induced $G_{q/11}$ activation, important GPCRs known to activate RhoA signaling include histamine-1 receptor (H_1 receptor) [278,279] and sphingosine-1-phosphate receptor ($S1P_3$ receptor) [280], whose ligands both control endothelial cell permeability.

Interestingly, not only receptors of the GPCR family are able to activate Rho-GTPases, but also tyrosine kinase receptors such as the ephrin receptor, which senses membrane-bound ligands (ephrins) of neighboring cells during the formation of cell contacts and subsequently activates RhoA. In this respect, ephexins that are directly coupled to the ephrin receptor act as RhoGEFs for RhoA [281]. In the vascular system, Ephexin5 (ARHGEF15, alternative name: Vsm-RhoGEF, Vascular smooth muscle RhoGEF) expressed in vascular smooth muscle cells regulates contractility of this cell type by modulating the actin cytoskeleton in response to the formation of cell–cell contacts with other adjacent smooth muscle cells [282]. Interestingly, other ephexins such as Ephexin1 (ARHGEF27, alternative name: NGEF, Neuronal guanine nucleotide exchange factor), Ephexin2 (ARHGEF19, alternative name: WGEF, Weakly similar to Rho GEF), and Ephexin3 (AHRGEF5, alternative name: TIM, Transforming immortalized mammary) have also been shown to activate RhoA in neuronal, intestinal, and cancer cells [216,230,231,235].

In addition to receptor-mediated Rho activation, there are also specialized RhoGEFs that trigger highly localized signaling to Rho in response to specific cell structures. One example is the microtubule-associated RhoGEF GEF-H1 (AHRGEF2), which is activated upon depolymerization of microtubules [283]. This is particularly the case when Toll-like receptor 4 (TLR4) is activated in response to endotoxins, thereby modulating the immune response and barrier function of endothelial cells [214,284–286]. Another example is the RhoA, RhoB, and RhoC-activating RhoGEF TEM4 (tumor endothelial marker 4, ARHGEF17), which is associated with the cadherin-catenin complex at cell junctions and serves as an actin-binding protein [229,287]. Because TEM4 is a junctional protein, it is also an important regulator of endothelial barrier function [287], but TEM4 has also been shown to regulate endothelial cell migration and suppress cell contraction [288]. Similarly, the RhoA-specific RhoGEF Syx (synectin-binding RhoA exchange factor, also termed PLEKHG5, pleckstrin homology and RhoGEF domain containing G5) is localized at tight junctions and also regulates endothelial barrier function [239]. In addition, Syx, expressed in both splice variants Syx1 and Syx2, is also involved in the control of endothelial cell migration [289,290] and angiogenesis [291]. Syx also interacts with the Crumbs complex, which is involved in the establishment of cell polarity, which is also important for directed migration and growth during angiogenesis [292]. Another factor in the activation of RhoA, the Rho/rac-specific RhoGEF VAV1, which is expressed in hematopoietic cells but also in endothelial cells, has been shown to associate with δ-catenin (neuronal catenin, but also expressed in endothelial cell) in cell–cell junctions of endothelial cells and to regulate cell migration [293]. Interestingly, VAV1 is upregulated in hypoxic endothelial cells and stabilizes hypoxia-induced transcription factor 1a (HIF1a) to ultimately promote the vascular response to hypoxia [294]. In *in vivo* experiments, endothelial cells deficient of Vav2 and Vav3, the two siblings of Vav1, displayed reduced migration and neovascularization in tumor growth [295]. In this context, activation of Rac, but not RhoA, by Vav2, triggered by VEGF signaling via VEGFR-2 and the kinase Src (cellular sarcoma), is known to control endothelial cell permeability [296]. In contrast to TEM4, Syx, and VAV1, the RhoA-, RhoB-, and RhoC-activating p190RhoGEF (AHRGEF28) localizes to focal adhesions and is involved in focal adhesion formation and cell migration [297]. In addition, p190RhoGEF has been shown to be involved in stretch-induced spatial reorientation of migrating endothelial cells [298] and in the regulation of tumor angiogenesis via focal adhesion kinase (FAK) [299]. Another important factor in stretch-induced reorientation of cells is the RhoA-activating RhoGEF SOLO (ARHGEF40) [298], which is known to bind to intermediate filaments (keratin-8/keratin-18) and is crucial for the response of the actin cytoskeleton to mechanical forces [238,300]. Spatiotemporal signaling of RhoA triggered by RhoGEF ECT2 (epithelial cell transforming 2), together with LARG, is involved in endothelial cell barrier maintenance during transendothelial migration of leukocytes (diapedesis) [301]. Moreover, ECT2 is involved in VEGF-induced sprouting and migration of endothelial cells and thus is able to promote the process of angiogenesis with the help of the actin-binding protein KLEIP (Kelch-like ECT2 interacting protein) [302]. ECT2 has also been shown to cluster at the equatorial cell cortex during RhoA-regulated cytokinesis [303]. During the interphase of the cell cycle, ECT2-induced RhoB signaling in the nucleus was also shown to be involved in the control of apoptosis after cell injury [219]. In this context, RhoGEF NET1 (neuroepithelial transforming protein 1), localized in the nucleus, is additionally involved in the activation of RhoA and RhoB signaling to regulate DNA damage responses [219,304]. RhoGEFs localized in the nucleus are not available for activation of RhoA and RhoC in the cytosol. However, after phosphorylation, Net1 translocates to the cytosol [218], where it can activate RhoA and RhoC to promote cell survival and motility [218,220].

6. RhoA-, RhoB-, and RhoC-related RhoGAPs in vascular (patho) physiology

RhoGAPs are proteins that accelerate the slow intrinsic GTPase activity of Rho GTPases to hydrolyze bound GTP to GDP, which also transitions the protein from its active to an inactive state [106]. Therefore, RhoGAPs can be seen as functional "opponents" of the Rho-activating RhoGEFs that have been discussed previously. The GAP domain in RhoGAPs is about 190 amino acids long and often contains a conserved catalytic arginine residue within a loop structure (arginine finger) that is inserted into the GTP binding site of the Rho GTPase upon Rho GAP binding [106]. Thereby, the transition state between GTP to GDP is stabilized which reduces the activation energy needed for GTP hydrolysis and catalytically increases the reaction speed [305]. Currently, 66 human RhoGAPs have been described, and similar to RhoGEFs, one RhoGAP is often capable of inactivating several different Rho GTPases [106,306]. A list of RhoGAPs that are known to inactivate RhoA, RhoB, and RhoC is shown in Table 3 (with references [307–366]). Similar to the RhoGEFs, RhoGAPs can be influenced by other signaling pathways to modulate Rho activity [367].

One important RhoGAP for the maintenance of vascular homeostasis is ARHGAP18. The gene has also been referred to as *SENEX* (Latin for "old man") because it also regulates endothelial cell senescence pathways induced by oxidative stress or impaired flow, which increases the resistance of endothelial cells to proinflammatory signals (anti-inflammatory phenotype) [368–370]. In endothelial cells, ARHGAP18 has been described to localize to cell junctions in response to pro-angiogenic signals (e.g., VEGF) [335], to protrusions in migrating cells, to membrane ruffles, perinuclear regions, and microtubule-derived filamentous structures [371]. ARHGAP18 has been shown to suppress RhoA activity when overexpressed, whereas knockdown of ARHGAP18 leads to an increased RhoA activity which induces stress fiber formation, reduced migration, and impaired normal cell spreading of epithelial-derived cell lines [334]. In contrast, although knockdown of ARHGAP18 in endothelial cells led to increased formation of stress fibers, it also increased cell migration and promoted sprouting angiogenesis, effects that appeared to be triggered by increased RhoC activity rather than increased RhoA activity [335]. Similarly, Arhgap18 knockout mice displayed increased aortic ring sprouting *ex vivo* and more pronounced retinal angiogenesis, but also exhibited impaired, less dense vascular endothelial cell junctions [335]. siRNA-mediated depletion of ARHGAP18 also destabilized microtubule networks by decreasing both acetylated and detyrosinated tubulin [371]. Most interestingly, ARHGAP18 has been shown to protect against thoracic aortic aneurism by reducing proinflammatory pathways in smooth muscle cells [372]. In addition, ARHGAP18 is required for endothelial cells to align actin stress fiber formation with the direction of laminar flow. Lack of this function resulted in a widespread atherosclerotic phenotype in ApoE (apolipoprotein E) / Arhgap18 double-knockout mice. [373]. In this context. endothelial cell alignment via Arhgap18 was again not regulated by RhoA, but by RhoC activity [374].

The DLC (deleted in liver cancer) RhoGAP family with its members

Table 3
RhoGAPs known to inactivate RhoA, RhoB, and RhoC. Gene names were assigned according to the NCBI database. RhoGAPs with known function in vascular cells are highlighted in bold.

Gene name	Common protein names	Involved in the regulation of …	RhoA	RhoB	RhoC
ARAP1	ARAP1, CENTD2	Tumorigenesis	[307]	–	–
ARAP3	**ARAP3, CENTD3**	**Lamellipodia formation, microtubule organization, focal adhesion**	[308]	–	–
ARHGAP1	Cdc42GAP, p50 RhoGAP	Proliferation, migration, and invasion of cancer cells	[309,310]	–	–
ARHGAP4	p115 RhoGAP	Focal adhesion dynamics, migration, and invasion of cancer cells	[311–313]	–	–
ARHGAP5	p190-B RhoGAP	Spreading and migration of cancer cells	[314,315]	[315,316]	[315]
ARHGAP6	–	Actin reorganization, cell proliferation	[317,318]	–	–
DLC1	**ARHGAP7, p122-RhoGAP, StarD12**	**Endothelial cell focal adhesion assembly, proliferation, migration, angiogenesis**	[319]	[319]	[319]
ARHGAP8	BPGAP1	Pseudopodia formation, migration	[320,321]	–	–
ARHGAP9	10C	Cell adhesion	[322]	–	–
ARHGAP10	GRAF2, PsGAP	Tumorigenesis	[323,324]	–	–
ARHGAP11A	–	Cell cycle dependent oncogenesis, **Pulmonary arterial hypertension**	[325–328]	–	–
ARHGAP13	srGAP1	Epithelial junction maturation, contractility lamellipodia dynamics, cell migration	[329–331]	–	–
ARHGAP17	Nadrin, Rich1	Astrocyte morphology, epithelial barrier	[332,333]	–	–
ARHGAP18	**MacGAP, Senex**	**Angiogenesis, stress fiber alignment to laminar flow, cell junctions, microtubule network, reduced inflammatory response**	[334]	–	[335]
ARHGAP19	–	Cytokinesis in lymphocytes and cancer cell migration	[336–338]		
ARHGAP21	*Often confused with AHRGAP10*	Insulin secretion, cytoskeletal regulation in cancer cells, cellular transport	[339,340]	–	[339]
ARHGAP23	–	Cell shape and migration	[341]		
ARHGAP24	FilGAP, **p73RhoGAP**	Epithelial tubulogenesis, adherent junction, polarity migration, tumorigenesis, **angiogenesis**	[342]	–	–
AHRGAP26	Oligophrenin-1-like, GRAF (GRAF1)	Stress fiber formation	[343]	–	–
ARHGAP28	–	Stress fiber formation	[344]	–	–
ARHGAP29	**PARG1**	**Endothelial barrier, tubulogenesis (blood vessel lumen), migration**	[345]	–	–
ARHGAP30	–	Tumorigenesis	[346]	–	–
ARHGAP32	Grit, p200RhoGAP, p250RhoGAP	Axonal outgrowth	[347]	–	–
ARHGAP35	**P190-A (P190)**	**Endothelial cell focal adhesion dynamics, cell junctions, barrier function**	[348]	–	[349]
STARD13	**ARHGAP37, DLC2**	**Endothelial cell focal adhesion dynamics, proliferation, migration, angiogenesis**	[350]	–	[351]
STARD8	ARHGAP38, DLC3	Tumorigenesis	[352]	[353]	–
OPHN1	ARHGAP41, Oligophrenin-1	Actin cytoskeleton, interneuron migration, myocardial inflammation & apoptosis	[354–356]	–	–
ARHGAP42	**GRAF3**	**Smooth muscle cell contractility, blood pressure**	[357]	–	–
ARHGAP45	**HMHA1**	**Endothelial barrier,** hematopoietic cells and cancer cell spreading & migraton	[358,359]	–	–
GMIP	ARHGAP46	Vesicle trafficking, cortical actin, neuronal migration	[360–362]	–	–
TAGAP	ARHGAP47	*T*-cell differentiation	[363]	–	–
MYO9A	Myosin-IXa, Myr7	Epithelial cell junctions and migration	[364]	–	–
MYO9B	Myosin-IXb CELIAC4, Myr5	Lamellipodia formation, cell shape & migration of immune cells	[365]	[365]	–
RACGAP1	**MgcRacGAP**	**Endothelial barrier function**, cytokinesis	[366]	–	–

DLC-1 (ARHGAP7 or StarD12,), DLC-2 (ARHGAP37 or StarD13), and DLC-3 (ARHGAP38 or StarD8) is known to be downregulated in a variety of cancers [375]. They are also members of the StarD (StAR-related lipid transfer domain protein) protein family because of their START (steroidogenic acute regulator-related lipid transfer) domain which allows the proteins to bind hydrophobic lipids and sterol-related membrane components (e.g. phosphatidylcholine / cholesterol) [376]. The RhoGAP domain of DLC-1 has been shown to induce GTP hydrolysis in RhoA, RhoB, and RhoC [319]. DLC-1–3 are concentrated at focal adhesions [352,377,378], where these RhoGAPs interact with Tensin1 or 2 [352,377–380], both actin- and integrin-linked scaffold proteins that transmit cellular traction forces to the extracellular matrix and serve as a platform for proteins that regulate the assembly and disassembly of focal adhesions [381]. Moreover, in areas outside focal adhesions, DLC-1 interacts with phosphorylated nonmuscle myosin IIa/b on actin bundles, possibly attenuating RhoA-induced stress fiber formation and contractility [382]. Decreased DLC-1 expression induces proliferation and colony formation of cancer cells, which is why it is considered a tumor suppressor [383]. In the context of pathologies associated with vascular stiffness, such as atherosclerosis or pulmonary arterial hypertension, DLC-1 expression has been found to be increased [384]. Moreover, it has also been shown that its GAP domain independently stabilizes local clusters of the adhesion molecule ICAM-1 in endothelial cells required for transendothelial migration of leukocytes (diapedesis), through which DLC-1 may also contribute to vascular inflammation when upregulated [385]. Further analyses show that knockdown of DLC-1 leads to impairment of angiogenic endothelial cell function [385,386]. Although decreased DLC-1 expression has been associated with endothelial dysfunction and the formation of angiosarcoma, recent results from endothelial cell-specific Dlc-1 knockout mice suggest that loss of Dlc-1 in the vascular endothelium does not compromise kidney and liver function and (in contrast to previous publications) is not associated with an increased risk of angiosarcoma formation in mice [387]. The postulated promoting role of DLC-1 in cancer-related angiogenesis may be attributable to the observation that reduced DLC-1 expression in healthy epithelial cells, similar to hypoxia in cancer cells, stimulates VEGF production, which then induces endothelial cell-related angiogenesis [388], an effect that may contribute to tumorigenesis in addition to the positive effects of DLC depletion on cell proliferation. Partially in contrast to DLC-1, it has been shown that silencing of DLC-2 reduces endothelial cell adherence but increases cell migration and angiogenesis *in vitro*, and that Dlc-2 knockout leads to increased tumor growth and associated tumor angiogenesis *in vivo* [389]. Interestingly, the endothelial phenotype induced by DLC-2 silencing was rescued by knockdown of RhoA, suggesting that increased RhoA activity may be mechanistically important [351]. Also, in non-vascular cells, such as human glioblastoma cells, hypoxia and EGF stimulation-mediated activation of RhoA and RhoC was shown to depend on the presence of DLC-2 (StarD13) [351]. In contrast, the function of DLC-3 in vascular cells has not yet been characterized. However, in cancer cells, DLC-3 has been shown to control RhoB-induced actin remodeling at endomembranes to affect membrane trafficking and sorting mechanisms required for invadopodia formation, which are responsible for matrix metalloproteases-mediated degradation of the extracellular matrix [353].

Another important RhoGAP is the PI3K and Rap1 (Ras-associated proximate 1) activated RhoGAP ARAP3 (ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3, alias name Centaurin Delta 3, CENTD3), which has a dual GAP activity towards RhoA and Arf6 (ADP ribosylation factor 6) [308]. This dual activity is mediated by two GAP-domains, one ArfGAP domain mainly regulated by phosphatidylinositol 3,4,5-trisphosphate (PIP_3 produced by PI3Kα) binding and one RhoGAP domain regulated by Rap1-GTP binding [83]. Thereby, the membrane-bound PIP_3 localizes ARAP3 to the plasma membrane and brings it into contact with activated RhoA and Arf6 to induce GTP hydrolysis and inactivation of RhoA [308,390]. Knockdown of ARAP3 has been shown to increase both Arf6 and Rho activity in fibroblasts, thereby impairing PDGF-induced lamellipodia formation, delaying the formation of protrusions and reorganization of the microtubule network, while not affecting the migration speed of cells [391]. Deletion of Arap3 in mice induced autonomous endothelial cell defects in the process of sprouting angiogenesis and thereby led to embryonic death at midgestation. Interestingly, these defects could be reproduced in knock-in mice expressing an Arap3 point mutant that cannot be activated by PIP_3, demonstrating the importance of PIP_3-related ARAP3 activity in vascular development [392]. In addition, ARAP3 can be recruited to the cell membrane near focal adhesions via the local synthesis of phosphatidylinositol-3,4-bisphosphate (PIP_2 produced by PI3K-C2β) to induce disassembly of focal adhesions by inhibiting RhoA activity [393]. In contrast to ARAP3, much less is known about its family member, the RhoA and Arf6 regulating RhoGAP ARAP1 [307], and therefore it is still unclear whether it is involved in the regulation of RhoA activity in vascular cells.

The p190 RhoGAPs, with their two proteins p190 (ARHGAP35, now referred to as p190-A) and p190-B (ARHGAP5), represent another family of endothelial RhoGAPs. In the resting state, p190 and p190-B are mainly localized to lamellipodia in endothelial cells and preferentially inactivate RhoA when activated [394]. Active p190 RhoGAP has a major role in early stages of cell adhesion when integrin binding to the extracellular matrix (integrin engagement) is followed by a rapid decrease in basal RhoA activity [395]. This process appears to be controlled by Src (cellular sarcoma)-dependent phosphorylation of p190 RhoGAP in response to integrin signaling [396], although other stimuli like folic acid can also induce Src-mediated phosphorylation of p190 RhoGAP [397]. Moreover, integrin-mediated activation of focal adhesion kinase (FAK) is also able to phosphorylate and activate p190 RhoGAP in endothelial cells, controlling RhoA activity during restoration of endothelial barrier function [398] as well as cell polarity and localization of p190 RhoGAP at focal adhesions or cell protrusions [399]. Interestingly, also a PKCδ-mediated phosphorylation of p190 RhoGAP has been suggested to control RhoA activity in regulating endothelial barrier function and focal adhesion and stress fiber formation [400]. In this regard, p190 RhoGAP was also shown to interact with p120-catenin (catenin δ1), which recruits p190 RhoGAP to the cell periphery and locally inhibits RhoA to stabilize VE-cadherin-mediated cell junctions in endothelial barrier function [401]. In addition, during initial cell spreading of endothelial cells, p190 RhoGAP is enriched in lipid rafts to control RhoA activity, a process which is coordinated by Filamin in response to cytoskeletal structure [402]. Furthermore, the disassembly of the cytoskeleton in response to collagen XVIII-derived angiogenesis inhibitor endostatin is also mediated by p190 RhoGAP, whose Src-dependent phosphorylation is induced by integrin-interacting Caveolin-1 [403]. Integrin/Calveolin-1/Src-mediated signaling is also relevant to the control of RhoA activity by p190 RhoGAP in endothelial cells exposed to shear stress [395]. Interestingly, in Hela cells, p190 RhoGAP was also found to act as an antagonist in RhoGEF ECT2-mediated regulation of cytokinesis [404]. In neuronal cells, p190 RhoGAP was shown to associate with plexin, an important receptor for semaphoring signaling [405]. In contrast to p190 (p190-A), much less is known about the endothelial functions of p190-B, a homologous protein that acts similarly to p190 but is regulated differently in endothelial cells [394,401]. Deficiency of p190-B RhoGAP has a strong tumorigenic potential [406] and within the brain, endothelial p190-B contributes to blood brain barrier disruption upon meningitic *Escherichia coli* infection [407]. Similar to p190 RhoGAP, p190-B also appears to have important functions in preserving the endothelial barrier. Indeed, mutations of p190-B have been shown to be involved in the lethal systemic capillary leak syndrome (Clarkson's disease), which surprisingly causes increased and sustained RhoB activation in dermal microvascular endothelial cells that disrupts barrier function [316]. In addition, p190-B has been shown to be involved in podosome formation, a morphological phenomenon known from immune cells that can be induced in endothelial cells by certain factors, e.g. VEGF-A [408]. Similar to invadosomes in cancer

cells, endothelial podosomes carry transmembrane matrix-metalloproteases (e.g. MMP14) that degrade extracellular matrix to allow invasion into surrounding tissues during angiogenesis. In this respect, it has also been shown that both p190 and p190B RhoGAP are involved in the direct regulation of matrix metalloprotease expression, including MMP14 and MMP2 [394].

Another important RhoGAP in vascular cells is ARHGAP29, which is regulated by Rap1 activity [409]. In endothelial cells, Rap1 effector Radil (Rap associating with DIL domain) induces the recruitment of ARHGAP29 to the plasma membrane, while Rap1 effector Rasip1 (ras-interacting protein 1) presumably regulates ARHGAP29 activity [410]. Activated ARHGAP29 then suppresses local RhoA activity to induce cell spreading of endothelial cells and to stabilize the barrier function of the vascular endothelium [409,410]. Rasip1-driven ARHGAP29 is also required for endothelial cell tubulogenesis, a process necessary for the formation of the lumen of developing blood vessels that requires tightly regulated cytoskeletal architecture, cell junctions, and focal adhesions [411,412]. During tubulogenesis in embryonic development, suppression of RhoA activity by ARHGAP29 is critical for reducing actomyosin contractility to allow controlled expansion of the vascular lumen [413]. In addition to Rasip1, the Rap1 effector and actin-binding protein Afadin can activate ARHGAP29. Similar to Afadin knockdown, also the knockdown of ARHGAP29 has been shown to reduce endothelial cell migration and tube formation *in vitro* [345].

A very important RhoGAP for the vasculature is the smooth muscle-selective ARHGAP42, which is also termed GRAF3 (GTPase regulator associated with focal adhesion kinase-1). GRAF3 activity is regulated by Src- and FAK-mediated phosphorylation but also presumably by dynamic allosteric auto-inhibitory mechanisms [414]. siRNA-mediated knockdown of GRAF3 in cultured smooth muscle cells increased and prolonged RhoA activation upon sphingosine 1-phosphate treatment and induced extended stress fiber and focal adhesion formation [415]. Interestingly, mice in which Graf3 was deleted showed increased blood pressure and pressure response (aortic contraction) to angiotensin II, suggesting that Graf3 limits RhoA-dependent smooth muscle cell contractility [415]. Re-expression of Graf3 in these animals was able to restore blood pressure to normal [416]. In addition, Graf3-depleted mice also showed higher expression of several genes involved in enhanced smooth muscle contractility, whereas Graf3 mRNA in vascular smooth muscle cells was regulated by increases in blood plasma volume and by TGFβ1 (transforming growth factor β1), suggesting that Graf3 may act as a blood volume-sensitive regulator to control smooth muscle contractility and systemic blood pressure [357]. Another mouse model that transiently and modestly increased Graf3 expression in vascular smooth muscle cells caused a stable drop in blood pressure of about ~ 10 mmHg compared with control animals [414], opening an interesting perspective for allosteric regulation of GRAF3 as a novel strategy in antihypertensive therapy. In humans, mutations that impair GRAF3/ARHGAP42 function have been associated with systemic hypertension as well as interstitial lung disease [417]. For example, a regulatory element comprising the ARHGAP42 single nucleotide polymorphism rs604723, which has strong SMC-selective allele-specific activity, has been shown to be associated with systemic hypertension in humans and can lead to a decrease in ARHGAP42 expression [416]. Indeed, the minor T allele at rs604723 promotes SRF (serum response factor) binding and deletion of this regulatory element in cultured human SMCs markedly reduced endogenous ARHGAP42 expression. Interestingly, in an apatinib-induced hypertension model in rats, a dose-dependent decrease in Graf3 mRNA expression and increased vascular remodeling was observed, whereas RhoA and RockII (but not RockI) expression increased, especially in the mid aorta [418].

To date, only few publications are available on the impact of other RhoGAPs on vascular homeostasis and function. These include p73 RhoGAP (ARHGAP24, alternative name: FilGAP, Filamin A binding RhoGAP), which has been described as an important regulator of the angiogenic capacity of endothelial cells. Indeed, knockdown of p73 using virally transduced anti-sense constructs increased RhoA activity and inhibited angiogenesis *in vivo* as well as migration, proliferation, and tube formation of endothelial cells *in vitro* [419]. However, further research on p73 RhoGAP has so far tended to focus on other cell types. Similarly, the RhoGAP ARHGAP45 (also referred to as HMHA1, minor histocompatibility antigen 1), which has been primarily associated with the regulation of RhoA activity in T cells, has also been shown to be expressed in endothelial cells, where it is involved in the regulation of endothelial barrier function [359]. However, the role of HMHA1 in this process appears to be mainly related to its GAP activity towards Rac1 and not RhoA. Although not yet studied experimentally in this context, multiple microarray analysis on differentially expressed genes identified ARHGAP11A as a potential therapeutic target in patients with pulmonary arterial hypertension [327]. In addition, RACGAP1 (MgcRacGAP), which also appears to possess RhoGAP activity, has also been shown to regulate melanoma-induced transendothelial migration, focal adhesion formation, endothelial barrier function, and the recovery phase after thrombin-induced endothelial permeability [366,420].

7. RhoGDIs in the regulation of RhoA/B/C function in the vascular system

Compared to the large number of RhoGEFs and RhoGAPs, only three RhoGDIs are known [421]. Interaction of Rho GTPases with an RhoGDI converts the protein into a cytosolic pool of Rho GTPases that is long-term inactivated, long-term stable, but also rapidly reactivatable [422]. RhoGDIs bind RhoA/B/C at their switch I and II regions and prevent the flexible transition of the GTPase from the inactive to the active state. In addition, they act as chaperones by preserving the proper folding of the protein and preventing proteasomal degradation [90,423]. Dissociation of Rho GTPases from RhoGDIs can be regulated by phosphorylation of the RhoGDI, interactions with proteins that act as protein displacement factors or by phospholipids with regulatory functions [424,425]. The most abundant RhoGDI is the ubiquitously expressed RhoGDI-1 (also known as RhoGDIα) which interacts with Cdc42 [426] but also with RhoA and RhoC [427]. In contrast, RhoGDI-2 (also known as RhoGDIβ) is mainly expressed in hematopoietic cells but can also be found in endothelial cells [428]. The weakly expressed RhoGDI-3 (RhoGDIγ) has a unique *N*-terminal extension that allows the protein to reside in both the cytoplasm and the Golgi apparatus [429]. A recent full study of RhoGDI targets was able to show that RhoA interacts with both RhoGDI-1 and RhoGDI-3, whereas RhoB interacts only with RhoGDI-3 and RhoC interacts with all RhoGDIs [430]. It is worth mentioning that some of these interactions could not be observed *in vivo* [429,431]. Surprisingly little is known about the regulation of Rho activity by GDIs in vascular cells. One problem with GDI research is that it is usually impossible to clearly attribute the observed effects to a particular Rho GTPase, as too many of them may be affected simultaneously. However, in the vascular system, RhoGDI-1 has been shown to modulate RhoA activity in the regulation and maintenance of endothelial barrier function [427,432,433]. In contrast to its role as a negative regulator of Rho activity, RhoGDI-2 has also been postulated to positively regulate Rho activity in immune cells during transendothelial migration [434]. However, for endothelial cells, downregulation of RhoGDI-2 was shown to explain the positive effects of CIF1 (CR6-interacting factor 1) on endothelial cell migration, a mitochondrial factor shown to inhibit RhoGDI-2 expression in this cell type [428]. Similarly, in vascular smooth muscle cells, the AT_1 receptor was shown to promote both RhoGDI-1 and RhoGDI-2 degradation, contributing to vascular smooth muscle cell proliferation and vascular remodeling [435].

8. Conclusion

RhoA, RhoB, and RhoC can transduce signals via many different effector molecules that are important for the regulation of various

functions in vascular cells. In this context, these Rho GTPases are themselves subject to complex regulation involving many different RhoGEFs and RhoGAPs, which in turn control local Rho activity at specific structures of the cell and coordinate their spatiotemporal activation and inactivation. Most research on the interactions of Rho with effectors, GEFs, GAPs, and GDIs has been conducted with a focus on RhoA, one of the best-studied Rho GTPases, which means, however, that the understanding of RhoB and RhoC and their influence on vascular homeostasis and pathology still has major gaps that need to be filled by future research. Although their protein structure is very similar, it has been shown that there are major differences between RhoA, RhoB, and RhoC in terms of gene expression, posttranslational modification, localization, activity regulation, and interaction with specific RhoGEFs, RhoGAPs, and RhoGDIs. For example, although this topic has been studied for decades, the interplay of RhoA- and RhoC in cellular processes such as stress fiber formation, focal adhesion dynamics, and cell–cell junction control, as well as cell migration, is still poorly understood. Moreover, RhoA/B/C do not act alone, but often in concert with other Rho GTPases such as Cdc42 or Rac1, making it even more difficult to unravel the full complexity of their actions. Future research efforts could therefore focus on the individual elucidation of Rho-mediated functions in the vasculature, using the knowledge accumulated so far on Rho GTPases, GEFs, GAPs, and effectors in integrative research approaches. This could help to understand how the local Rho activity of different Rho GTPases is regulated and how it can be targeted therapeutically, for example, to favorably influence the course of cardiovascular disease or cancer [436]. For instance, for the treatment of hypertension, modulation of Rho GTPase actions on vascular smooth muscle cell contractility and arterial or peripheral vascular resistance could be considered [437]. These strategies could include direct pharmacological manipulation of Rho effectors such as ROCK or their downstream targets, strategies that are currently being tested in clinical trials in the context of various vascular diseases [437]. In addition, strategies targeting RhoA directly, which include GTP-binding inhibitors [438], or known toxins that act as selective non-covalent allosteric RhoA inhibitors or block its plasma membrane localization may be of interest [437]. Finally, also strategies targeting RhoGEFs and RhoGAPs known to be involved in hypertension could represent promising therapeutic approaches. These include the inhibition of the GEF activity of RhoGEFs p115-RhoGEF, PDZ-RhoGEF, LARG, and p63-RhoGEF that are known to be involved in regulation of vascular smooth muscle contractility in hypertension [437]. Moreover, therapeutic enhancement of RhoGAP function of GRAF3, which also regulates vascular smooth muscle contractility and blood pressure homeostasis, could lead to antihypertensive effects and beneficial effects in cardiovascular disease [437].

CRediT authorship contribution statement

Robert Eckenstaler: Writing – original draft, Visualization. **Michael Hauke:** Writing – original draft. **Ralf A. Benndorf:** Writing – original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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