Atypical RhoUV GTPases in Development and Disease

Stephanie Woo[1,2] and Leesa Strasser[1,2], [1]Department of Molecular Cell Biology, [2]Quantitative and Systems Biology Graduate Program, University of California, Merced, CA USA

Abstract

RhoU and RhoV are members of the Rho family of small GTPases that comprise their own subfamily. RhoUV GTPases are classified as atypical due to the kinetics of their GTP/GDP binding cycles. They also possess unique N- and C- termini that regulate their subcellular localization and activity. RhoU and RhoV have been linked to cytoskeletal regulation, cell adhesion, and cell migration. They each exhibit distinct expression patterns during embryonic development and diseases such as cancer metastasis, suggesting they have specialized functions. In this review, we will discuss known functions of RhoU and RhoV, with a focus on their roles in early development, organogenesis, and disease.

Key words: RhoU, RhoV, Rho GTPases, cell adhesion, cell migration, EMT, embryonic development, cancer, viral infection

Introduction

Regulation of Rho GTPases

The Rho family GTPases are essential regulators of many cellular processes including cytoskeletal dynamics, cell migration, cell adhesion, proliferation, and apoptosis [1]. Rho proteins are part of the larger Ras superfamily of small G proteins and are distinguished by a Rho-specific insert within their GTPase domain [2]. Like all G proteins, Rho GTPases act as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. In the active state, Rho proteins undergo a conformational shift to allow specific effectors to bind, activating downstream signals for various cell processes [3] Cycling between the active, GTP-bound state and inactive, GDP-bound state is facilitated by a large number of regulatory proteins. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting the exchange of GDP for GTP [4]. GTPase-activating proteins (GAPs) inactivate Rho proteins by accelerating their intrinsic GTP hydrolysis activity [5].

In addition to nucleotide binding state, Rho GTPases are also regulated by subcellular localization. Rho proteins are post-translationally lipid modified on their C-terminal ends, primarily via prenylation, which enables localization to cell membranes where activating GEFs and downstream effectors are located [6]. A class of regulatory proteins called Guanine Dissociation Inhibitors (GDIs) can bind and mask these lipid modifications, sequestering the Rho proteins in the cytoplasm and preventing non-specific activation by membrane-localized GEFs [7]. In addition, GDI binding prevents nucleotide exchange, which locks the Rho protein in an inactivated state [8, 9]. Thus, GDIs function to maintain a constant cytoplasmic pool of inactivated Rho GTPases poised for rapid activation [7]. However, GDIs have also been

demonstrated to actively extract Rho GTPases from cell membranes [9, 10], suggesting that GDIs may function beyond just passive sequestration.

Structure and Function of RhoU and RhoV

In mammals, there are 20 Rho family members of which the most extensively characterized are RhoA, Rac1, and Cdc42 — the so-called "classical" Rho GTPases [1]. Rho family members are grouped into different subfamilies based on amino acid sequence homology [11]. The RhoUV subfamily is composed of RhoU, also known as Wnt-1 responsive Cdc42 homolog (Wrch1), and RhoV, also known as Cdc42 homologous protein (Chp) or Wrch2 [12]. This subfamily has been proposed to be derived from Cdc42 [11]. RhoU shares 57% sequence homology to Cdc42 [13] while RhoV is 52% homologous to Cdc42 [14]. Both RhoU and RhoV are structured similarly (Fig. 1). They consist of a central G domain that is mostly conserved with other Rho GTPases, but their N- and C- terminal ends significantly diverge from classical Rho GTPases. The N-terminus contains a polyproline domain that can bind SH3 domain-containing adaptor proteins [13–17], while the C-terminus contains unique sequences that direct subcellular localization (discussed below).

Compared to classical Rho GTPases, RhoU and RhoV exhibit elevated rates of GDP/GTP exchange [15, 16]. This difference in nucleotide cycling may be due to a key amino acid residue within the nucleotide binding site (Fig. 1). Classical Rho GTPases contain a phenylalanine at position 28 (F28), but RhoUV and other "atypical" Rho GTPases contain a tyrosine at this same position [18]. This residue difference is reminiscent of the "fast cycling" F28L H-Ras mutants, which exhibit a high rate of GDP/GTP exchange and can function as constitutively active mutants [19]. Thus, RhoUV proteins are also assumed to predominantly exist in an activated state [12].

Regulation of membrane localization also differs between RhoUV and classical Rho GTPases. Rho UV proteins have been shown to localize to both the plasma membrane and endosome compartments [20–22]. This membrane localization is dependent on posttranslational lipid modification of their C-terminal ends, as with other small G proteins (Fig. 1). However, unlike classical Rho GTPases, RhoU and RhoV are palmitoylated rather than prenylated [20, 21, 23], which is notable as palmitoylation is a reversible process while prenylation is not. Moreover, RhoUV proteins have additional C-terminal sequences besides the palmitoylation motif that also function in membrane localization [22, 23].

Together, the unique features of RhoUV GTPases in terms of nucleotide cycling and membrane localization have led to the suggestion that RhoUV proteins are primarily regulated by their subceullular localization rather than by control of nucleotide binding state by GEFs or GAPs [20, 21]. Consistent with this idea, RhoV's ability to induce lamellipodia and localize to the Golgi apparatus was shown to require its C-terminal domain, which includes the residues and motifs necessary for membrane localization [14]. In addition, RhoGDI-3 was shown to regulate of RhoV activity by chaperoning RhoV to different cellular compartments [24]. On the other hand, both RhoU and RhoV have been shown to interact with β-Pix [25, 26], a known Rho GEF [27]. Thus, additional work is likely needed to fully understand how RhoUV GTPases are regulated in cells.

Downstream Effectors RhoUV Proteins

RhoUV proteins have been implicated in many different cellular processes including cell adhesion, migration, polarity, proliferation and survival, and gene expression [12]. Several effectors have been identified that mediate these processes downstream of RhoU and RhoV (Fig.

2). Many of these effectors belong to the p21-activated kinase (PAK) family of serine/threonine kinases including PAK1 [28, 29], PAK2 [14], PAK4 [28, 30], and PAK6 [31].

PAK1 is known to regulate cell adhesion by forming a multiprotein complex with the GEF β -PIX and the focal adhesion protein paxillin [27], so not unexpectedly, a major outcome of RhoUV-PAK signaling appears to be the regulation of cell adhesion. In zebrafish embryos, both RhoV and RhoU were shown to interact with both PAK1 and β-PIX to control cell adhesion [25, 26]. RhoU was shown to regulate paxillin phosphorylation and focal adhesion dynamics via PAK4 in breast cancer cells [30]. Furthermore, RhoU was reported to localize to osteoclast podosomes and to focal adhesions in HeLa cells and fibroblasts, and localization to adhesive structures required the PAK-binding effector loop and C-terminal extension of RhoU [32].

PAK activation downstream of RhoUV can also regulate cytoskeletal dynamics and actin-driven protrusion. PAK2 binding to RhoV was suggested to induce lamellipodial protrusion [14], and RhoU signaling through PAK1 and Jun kinase 1 (JNK1) could induce actin rearrangements and filopodia formation [13]. The JNK pathway was also linked to RhoV and PAK6 during induction of apoptosis in PC12 and HEK298 cells [33].

RhoUV proteins have also been linked to growth factor receptor tyrosine kinase signaling, primarily through the N-terminal SH3-binding domain. In breast cancer cells, RhoV was shown to directly bind to Grb2, an SH3 domain-containing adapter protein that functions downstream of the epidermal growth factor receptor (EGFR) [34]. Disrupting the binding between RhoV and Grb2 inhibited EGF-dependent migration. RhoU has also been linked to EGRFR signaling through Grb2, which was shown to activate JNK/AP1-dependent transcription and cell motility [17].

Other known effectors of RhoU include the protein tyrosine kinase Pyk2, which promoted filopodia formation [35], and the cell polarity protein Par6, which facilitated tight junction formation in epithelial cells [36]. Other potential RhoV effectors include N-WASP, MLK3, and Par6 [28]; however, these effector candidates were identified by immunoprecipitation, and the functional significance of these interactions has not been demonstrated.

RhoU and RhoV in Development

Early development

Both RhoU and RhoV have been reported to be expressed in several vertebrate embryos at very early developmental stages, i.e., prior to organogenesis. In chick embryos, *cRhoV* and *cRhoU* are expressed in the primitive streak and Hensen's node at Hamburger-Hamilton (HH) stage 5, with *cRhoU* also present in the prospective anterior neural plate [37]. In *Xenopus* embryos, both RhoU and RhoV expression are observed in the early gastrula— RhoU is expressed within the dorsal marginal zone, neural plate border, and pharyngeal arches [38, 39] while RhoV is expressed in the dorsal marginal zone and within involuting mesodermal cells [40].

In zebrafish embryos, *rhov* expression was reported to begin as early as 5 hours post-fertilization (hpf) [25]. Knockdown of *rhov* blocked the ability of embryos to undergo epiboly — the process by which blastomeres spread over and eventually cover the yolk. These epiboly defects were shown to be due primarily to mislocalization of E-cadherin and β-catenin away from adherens junctions via a mechanism that also required β-pix and PAK1.

The Wnt signaling pathway is known to be very active during development and is involved early symmetry breaking and axis specification events [41]. Although a role for RhoUV

proteins has not yet been reported for these early developmental processes, RhoU and RhoV are known to respond to Wnt signaling. RhoU was initially identified as a Wnt-responsive factor [13]. In mouse embryo fibroblasts, RhoU was shown to be transcriptionally regulated by Wnt-1 in a β-catenin-independent, JNK-dependent manner [42]. And in *C. elegans*, loss of the RhoU/V ortholog CHW-1 resulted in uniform distribution of Wnt receptors in vulval precursor cells, leading to an inability to establish apicobasal polarity [43].

Heart Development

A study in zebrafish embryos has suggested a role for RhoU in cardiac development [26]. Due to a genome duplication event, zebrafish possess two RhoU genes, *rhoua* and *rhoub*. Expression of *rhoua* was detected in the developing heart tube by 36 hpf; expression then became progressively restricted to the atrioventricular canal between 48–72 hpf. Knockdown of *rhoua* resulted in abnormalities in the atrioventricular canal and aberrant cardiac looping. *rhoua*-deficient cardiomyocytes also exhibited reduced expression and mislocalization of the adhesion proteins N-cadherin and Alcama, which depended on *pak1*and *arhgef7b*, the zebrafish homolog of β-pix.

Gastrointestinal Development

Multiple reports have suggested a role for RhoU and RhoV in development of the gastrointestinal system. In gastrointestinal organs, the inner epithelial layers are derived from the endoderm while the surrounding smooth muscle is derived from the mesoderm. In chick embryos, *cRhoV* was found to be expressed in the endoderm-derived layers of the foregut, caudal hindgut, gizzard and cloaca while *cRhoU* was broadly expressed throughout the mesoderm-derived layers of the GI tract except for the colon [37].

In mouse embryos, *RhoU* is expressed in the foregut epithelium during early somite stages, and its expression decreases once the epithelium develops into multiple layers [44]. Knockdown of *Rhou* in embryonic stem (ES) cells resulted in decreased expression of endoderm markers, indicating that RhoU facilitates endoderm differentiation. Mouse embryos produced from these *Rhou*-deficient ES cells exhibited a collapsed foregut and irregular thickness of the epithelium. Additionally, these *Rhou*-deficient mice exhibited decreased F-actin and α-tubulin levels within the apical domain of these epithelial cells. Interestingly, this study did not observe any defects in apicobasal polarity or Cadherin localization, in contrast to what was observed in zebrafish [25, 26].

Neural Crest Cells

Neural crest cells are a population of multipotent stem cells that arise from the dorsal neural tube at the boundary between the neural and nonneural ectoderm. During development, the neural crest cells undergo an epithelial-to-mesenchymal transition to migrate out of the neural tube and into several locations within the embryo as they differentiate into a wide variety of cell types [45]. Evidence suggests that RhoU regulates the migration of neural crest cells. In both chick and *Xenopus* embryos, RhoU is expressed in migrating neural crest cells [37, 39]. Overexpression of RhoU in *Xenopus* neural crest cells promoted extensive lamellipodial protrusions, and both overexpression and knockdown of RhoU inhibited proper cranial neural crest migration [39], suggesting that balanced levels of RhoU activity are required for optimal migration behavior.

Rather than regulating cell migration, RhoV may act to promote neural crest fate specification. In chick embryos, cRhoV expression in the neural folds resembles that of Wnt6 [37], a known neural crest inducer [46]. In *Xenopus* embryos, RhoV is initially expressed in the

neural crest progenitor domain, but its expression decreases once neural crest cells start migrating [40]. Overexpression of RhoV in led to an expansion of neural crest progenitors while loss of RhoV reduced expression of neural crest marker genes Sox9, Sox10, Slug, and Twist. Interestingly, expression of these neural crest markers could be rescued by RhoU expression. In contrast, RhoV was not able to rescue the neural crest cell migration defects seen in RhoU deficient embryos [39]. Together, these reports suggest that RhoU and RhoV may have distinct and partially overlapping functions in the neural crest.

RhoU and RhoV in Disease

Cancer

Many Rho GTPases are upregulated in tumors, including RhoU and RhoV [47]. RhoV has been shown to be highly expressed in lung adenocarcinoma (LUAD) tumors and was identified as a major predictor of poor prognosis in LAUD patients [48–50]. In A549 and PC9 lung cancer cell lines, RhoV overexpression increased cell proliferation, migration, and invasiveness [48, 49]. In a model of gefitinib-resistant lung cancer (PC9-GR), RhoV knockdown was shown to restored drug-induced apoptosis [48]. RhoV may also promote metastasis in lung cancer. Overexpression of RhoV in A549 cells was also shown to induce markers of epithelial-to-mesenchymal transition (EMT), including increased expression of Snail, Slug, and N-Cadherin coupled with decreased expression of E-Cadherin, while RhoV silencing suppressed EMT markers [49]. When these RhoV-deficient A549 cells were injected into nude mice, they produced significantly fewer metastases than control cells.

RhoV has also been identified as overexpressed in triple-negative breast cancer (TNBC), and its expression is correlated with metastasis and poor survival [34]. In various breast cancer cell lines, expression of a constitutively active RhoV mutant (G40V) increased cell migration

through a transwell assay while RhoV knockdown suppressed transwell migration, suggesting that RhoV may promote breast cancer invasiveness. These effects were dependent on EGFR signaling via binding of Grb2 to the SH3 domain of RhoV.

RhoU has also been implicated in several cancers including prostate cancer [51], breast cancer [30], and T-cell acute lymphoblastic leukemia (T-ALL) [52]. For T-ALL, RhoU was found to be upregulated in examined patient samples, and its expression was corelated with activated Notch signaling, which is often mutated in T-ALL. This study also demonstrated that RhoU could promote cell migration, adhesion to fibronectin, and F-actin content in cell line models of T-ALL.

Many prostate cancers exhibit overexpression of fatty acid synthase (FASN) and corresponding dysregulation of protein palmitoylation [53]. In a prostate cancer cell line, palmitoylation of RhoU was shown to be especially sensitive to FASN levels even though expression of total RhoU was unaffected [51]. In these cells, FASN-dependent palmitoylation of RhoU promoted phosphorylation of the focal adhesion protein paxillin, leading to increased cell adhesion turnover and cell migration. Interestingly, a very similar mechanism may operate in breast cancer cells. In MDA-MB-231 cells, RhoU was shown to promote cell migration, focal adhesion disassembly, and phosphorylation of paxillin in a PAK4-dependent manner [30]. Interestingly, PAK4 was also required in these cells to inhibit RhoU degradation.

As described above, many small GTPases including RhoU and RhoV are overexpressed in tumors, suggesting these proteins function as protooncogenes. In contrast, RhoU was reported to be downregulated in colorectal cancer. Loss of RhoU in a mouse model resulted in hyperplasia of the gut epithelium due to decreased apoptosis and increased proliferation, and a similar result was observed in cultured DLD-1 cells [54]. These results intriguingly suggest that RhoU may

possess tumor suppressor activity under specific contexts such as in the gut. Given that Wnt/beta-Catenin signaling is a major driver of colorectal tumors [55] and that RhoU is known to be Wnt responsive [13, 42, 43], it will be interesting to determine if RhoU interacts with the Wnt signaling pathway to suppress or enable tumor formation.

Viral Infection

Many Rho GTPases have been shown to be involved in the process of viral infection, primarily by promoting cytoskeleton rearrangements that make the cell more accessible for viral entry [56, 57]. In a cell culture-based screen, RhoV was identified as a host factor that enhanced entry of a subset of flaviviruses including Zika virus and dengue virus [58]. Because flaviviruses enter cells via receptor-mediated endocytosis and RhoV is known to localize to endosomal membranes [21], the authors investigated the effects of mutating the palmitoylation motif on RhoV (C234S). While some reduction in viral entry was observed, it was not consistent across experimental replicates. However, expression of GTPase-defective, constitutively GTP-bound RhoV mutant (G40V) did significantly reduce viral entry, suggesting that complete GTP/GDP cycling is necessary for RhoV to facilitate flavivirus infection.

Conclusion

Although not as extensively studied as RhoA, Rac1, and Cdc42, RhoUV GTPases are increasingly recognized as having important and unique functions. They appear to be especially critical for embryonic development (summarized in Table 1) and cancer progression (Table 2). When taken as a whole, published studies of RhoUV proteins have converged on a few key cellular processes regulated by these GTPases. One of these processes is the regulation of cell adhesion. In multiple cell types, RhoUV proteins have been demonstrated to regulate cell-cell adhesion and the localization of Cadherin receptors [25, 26, 49]. RhoUV proteins are also

implicated in cell-matrix adhesion [52] and the regulation of the focal adhesion proteins paxillin [30, 51]. Notably, paxillin can regulate stability of both integrin-dependent focal adhesions [27] and Cadherin-based adherens junctions [59], suggesting that RhoUV signaling may play a central role in coordinating cell-cell and cell-matrix adhesion.

Several lines of evidence point to a role for RhoUV proteins in regulating transitions between epithelial and mesenchymal cell states. As noted above, both RhoU and RhoV can regulate the levels and localization of E- and N-cadherin, key markers of epithelial and mesenchymal states, respectively [25, 26, 49]. RhoU and RhoV also play important roles in neural crest cells [39, 40], which prominently undergo EMT during their development. Both RhoU and RhoV are associated with cancer metastasis [30, 34, 48, 49], which often involves an EMT step as cells escape the primary tumor. Finally, RhoV has been shown to regulate the expression of the EMT transcription factors Slug and Twist in neural crest cells [40] and Snail, Slug, and Twist cancer cells [34]. The contribution of RhoU and RhoV to EMT/MET is a potentially impactful area for future investigation.

One underappreciated aspect of RhoUV proteins may be their specialized functions. Although similar in structure, RhoU and RhoV exhibit obvious differences in their spatiotemporal expression patterns, especially during development. RhoV expression is often more restricted in terms of developmental time points and cell and tissue types while RhoU is often expressed more broadly; this pattern is seen, for example, in the developing chick gastrointestinal tract [37]. This difference in expression pattern suggests that RhoV may be more specialized in function than RhoU. Consistent with this idea, RhoU was able to rescue RhoV loss of function in neural crest cells [40], but RhoV could not substitute for loss of RhoU [39]. In the future, it will be interesting to determine if this functional specialization broadly applies to other

contexts and cell types and, more importantly, to identify the mechanisms underlying the differences in RhoU versus RhoV function.

Perspectives

• RhoUV GTPases are relatively understudied but may have important and physiologically relevant functions that are distinct from classical Rho GTPases.

• RhoUV GTPases possess several distinct structural and functional properties, including divergent N- and C-terminal regions and increased GDP/GTP cycling. These atypical Rho GTPases have been shown to regulate the development of several different organ and tissue types and are implicated in diseases including several types of cancer and susceptibility to viral infection.

• Future work on RhoUV proteins should focus on in-depth characterization of the mechanisms underlying their function, especially in terms of coordinating cell adhesion and epithelial-to-mesenchymal transitions, as well as delineating the distinct functions of RhoU versus RhoV.

Figure Legends

Figure 1. Domain structures of RhoU and RhoV. RhoU and RhoV have several distinct features compared to the "classical" Rho GTPases. The N-terminus is expanded and contains a proline-rich domain (pink). The central G domain (blue) is mostly conserved except for a tyrosine residue at position 28 (Y28) instead of a phenylalanine (F28), which may underlie their rapid GDP/GTP cycling. At the C-terminal end, RhoUV proteins do not have a canonical CAAX sequence for prenylation. Instead, RhoU and RhoV are reversibly palmitoylated, for which there is no consensus sequence.

Figure 2. Downstream effectors and cellular processes regulated by Rho UV GTPases. SH3 domain-containing effectors such as Grb2 bind to the polyproline-rich domain (pink) at the N-terminus, while most other effectors are presumed to bind to the centrally located effector-binding domain (orange).

Tables

Table 1. RhoUV functions during development			
	Organism, Cell type	**Function**	**References**
RhoU	Zebrafish, cardiomyocytes	*Regulates localization of adhesion proteins, N-cadherin and Alcama. Regulates cardiac looping and formation of the atrioventricular canal.*	[26]
	Mice, foregut epithelium	*Facilitates endoderm differentiation. Regulates cytoskeletal organization and epithelial architecture.*	[44]
	Xenopus, neural crest	*Induces lamellipodial protrusion and cell migration.*	[39]
RhoV	Zebrafish, EVL	*Regulates localization of E-cadherin and β-catenin at cellular junctions*	[25]
	Xenopus, neural crest	*Induces expression of neural crest markers Sox 9, Sox10, Slug, Twist.*	[40]

Table 2. RhoUV functions in cancer.			
	Disease	**Function**	**References**
RhoU	Colorectal Cancer	*Downregulated in tumors. Maintains epithelial homeostasis by regulating apoptosis and proliferation.*	[54]
	Breast Cancer	*Overexpressed in tumors. Promotes cell migration and focal adhesion turnover.*	[30]

	Prostate Cancer	*Overexpressed in tumors. Promotes cell migration and focal adhesion turnover.*	[51]
	T-cell Acute Lymphoblastic Leukemia	*Overexpressed in cancerous cells. Promotes cells migration, adhesion and F-actin content.*	[52]
RhoV	Lung Adenocarcinoma Cancer	*Overexpressed in tumors. Promotes cell migration and proliferation. Promotes metastasis and EMT markers, e.g., downregulation of E-cadherin, upregulation of N-cadherin, Snail, Slug.*	[48, 49]
	Triple Negative Breast Cancer	*Overexpressed in tumors. Promotes migration and invasion.*	[34]

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